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(54) Title: RECOMBINANT CELLS THAT HIGHLY EXPRESS CHROMOSOMALLY-INTEGRATED HETEROLOGOUS GENES

(57) Abstract

Recombinant host cells are obtained that comprise (A) a heterologous, polypeptide-encoding polynucleotide segment, stably integrated into a chromosome, which is under transcriptional control of an endogenous promoter and (B) a mutation that effects increased expression of the heterologous segment, resulting in enhanced production by the host cells of each polypeptide encoded by that segment, relative to production of each polypeptide by the host cells in the absence of the mutation. The increased expression thus achieved is retained in the absence of conditions that select for cells displaying such increased expression. When the integrated segment comprises, for example, ethanol-production genes from an efficient ethanol producer like *Zymomonas mobilis*, recombinant *Escherichia coli* and other enteric bacterial cells within the present invention are capable of converting a wide range of biomass-derived sugars efficiently to ethanol.

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RECOMBINANT CELLS THAT HIGHLY EXPRESS CHROMOSOMALLY-INTEGRATED HETEROLOGOUS GENES

This application is a continuation-in-part of co-pending application Serial No. 07/352,062 (filed May 15, 1989), which is a continuation-in-part of application Serial No. 07/239,099 (filed August 31, 1988), now abandoned.

Work relating to this invention was supported in part by Grant FG05-86ER3574 from the Office of Basic Energy Science, U.S. Department of Energy, and in part by Grant 88-37233-3987 from the Alcohol Fuels Program, U.S. Department of Agriculture. The U.S. Government has certain rights in the invention.

Background of the Invention

The present invention relates to recombinant host cells that comprise a heterologous, polypeptide-encoding polynucleotide segment which is stably integrated into a chromosome and which is under control of an endogenous promoter. When the integrated segment comprises, for example, ethanol-production genes from an efficient ethanol producer like *Zymomonas mobilis*, recombinant *Escherichia coli* and other enterobacterial cells within the present invention are capable of converting a wide range of biomass-derived sugars efficiently to ethanol. This invention also relates to mutations that enhance production of proteins encoded by chromosomally-integrated, heterologous genes which are expressed under the control of an endogenous promoter, and to methods of identifying such mutations.

During glycolysis, cells convert simple sugars, such as glucose, into pyruvic acid, with a net production of ATP and NADH. In the absence of a functioning electron transport system for oxidative phosphorylation, at least 95% of the pyruvic acid is consumed in short pathways which regenerate NAD^+, an obligate requirement for continued glycolysis and ATP production. The waste products of these NAD^+ regeneration systems are commonly referred to as fermentation products.

Microorganisms are particularly diverse in the array of fermentation products which are specific for each genus. See, for example, Krieg, N.R., and J.G. Holt, eds. [1984] BERGEY'S MANUAL OF SYSTEMATIC BACTERIOLOGY (Williams & Wilkins Co., Baltimore). These products include organic acids, such as lactate, acetate, succinate, and butyrate, as well as neutral products like ethanol, butanol, acetone, and butanediol. Indeed, the diversity of fermentation products from bacteria has led to their use as a primary determinant in taxonomy. Krieg and Holt [1984], *supra*. End products of fermentation share several fundamental features. They are relatively nontoxic under the conditions in which they are initially produced but become more toxic upon accumulation. They are more reduced than pyruvate because their immediate precursors have served as terminal electron acceptors during glycolysis. The microbial production of these fermentation products forms the basis for our traditional and most economically successful applications of biotechnology and includes dairy products, meats, beverages, and fuels.

Most fuel ethanol is currently produced from hexose sugars in corn starch or cane syrup utilizing either *Saccharomyces cerevisiae* or *Zymomonas mobilis* (*Z. mobilis*). However, these are relatively expensive sources of biomass sugars and have competing value as foods. In addition, during fermentation much of the hexose is necessarily converted back to biomass,

comprising microbial cells, rather than to ethanol. For conventional ethanol-producing microorganisms, this biomass has limited commercial value at best, for instance, as a nutritional supplement, and therefore, represents inefficient utilization of the expensive sugar substrate.

Starches and hexose sugars represent only a fraction of the total carbohydrates in plants. The dominant forms of plant carbohydrate in stems, leaves, hulls, husks, cobs, etc., are the structural wall polymers, cellulose and hemicellulose. Hydrolysis of these polymers releases a mixture of neutral sugars which include glucose, xylose, mannose, galactose, and arabinose. No known organism in nature can rapidly and efficiently metabolize all of these sugars, particularly the pentoses, into ethanol or any other single product of value.

Escherichia coli (*E. coli*) and related enteric bacteria are the main commercially useful microorganisms that are capable of metabolizing the entire range of biomass-derived sugars by fermentation under anaerobic conditions. However, under anaerobic fermentation conditions, these organisms convert sugars to a mixture of soluble products, including small amounts of ethanol, that cannot be separated economically. See Ingram, L.O., T. Conway, D.P. Clark, G.W. Sewell, and J.F. Preston [1987] *Appl. Environ. Microbiol.* 53: 2420-2425. Thus, such enteric bacteria efficiently utilize the entire range of biomass-derived sugars but fail to produce a product of sufficient yield and uniformity to be commercially valuable.

Accordingly, there is a need for microorganisms which combine the efficient metabolism of the entire range of biomass-derived sugars, which is exhibited by certain enteric bacteria, such as *E. coli*, with the ability to produce high levels of a single, predominant, soluble fermentation product of commercial value, such as ethanol. Further, there is a continuing need for such organisms that can produce microbial biomass comprising

additional products, such as commercially valuable proteins, in sufficient yield and quality for economical recovery.

Fermentation pathways transform pyruvic acid into a mixture of acidic and neutral products. Two pathways dominate in enteric bacteria such as *E. coli*. Lactate dehydrogenase catalyzes the reduction of pyruvate to lactic acid, directly oxidizing NADH to NAD^+. The second pathway, involving pyruvate formate-lyase, is more complicated. Pyruvate formate-lyase, which catalyzes the cleavage of pyruvate to formate plus acetyl-coenzyme A, is a central enzyme of the anaerobic metabolism of *E. coli*, because under anaerobiosis this enzyme is responsible for metabolizing a large fraction of pyruvate. The *E. coli* gene encoding pyruvate formate-lyase (*pfl* gene) has been cloned and sequenced. See Christiansen, L., and S. Pedersen [1981] *Mol. Gen. Genet.* 181: 548-551; Rodel, W., W. Plaga, R. Frank, and J. Knappe [1988] *Eur. J. Biochem.* 177: 153-158. The *pfl* gene is preceded by multiple promoters, and it is induced to high levels of expression by anaerobiosis. See Sawers, G., and A. Bock [1988] *J. Bacteriol.* 170: 5330-5336.

The DNA used to provide ethanol-production genes for a recombinant host of the subject invention is isolated, for example, from *Z. mobilis*. This is a microorganism with unusual metabolic characteristics which is commonly found in plant saps and in honey. Wild-type *Z. mobilis* has long served as a natural inoculum for the fermentation of the *Agave* sap to produce pulque, a Mexican alcoholic beverage, and as an inoculum for palm wines. As noted above, this organism is also used for fuel ethanol production and has been reported to be capable of ethanol production rates which are substantially higher than those of yeasts.

Although *Z. mobilis* is nutritionally simple and capable of synthesizing amino acids, nucleotides and vitamins, the range of sugars metabolized by this

organism is very limited and normally consists of glucose, fructose and sucrose. Substrate level phosphorylation from the fermentation of these sugars is the sole source of energy for biosynthesis and homeostasis. *Z. mobilis* is incapable of growth without a fermentable sugar even in rich medium such as nutrient broth.

In *Z. mobilis*, two enzymes, pyruvate decarboxylase (PDC) and alcohol dehydrogenase, particularly form II (ADHII), are required to convert pyruvate to ethanol and regenerate NAD^+. High levels of the individual proteins are found in the cytoplasm of *Z. mobilis*, ranging from 2% to 5% each of the soluble protein. Such high levels are presumed to be essential for the high rates of NADH oxidation and glycolytic flux required for energy production. The cloning and sequencing of *Z. mobilis pdc* and *adhB* genes encoding PDC and ADHII, has been previously reported. See Conway, T., Y.A. Osman, J.I. Konnan, E.M. Hoffman, and L.O. Ingram [1987] *J. Bacteriol.* 169: 949-954; Conway, T., G.W. Sewell, Y.A. Osman, and L.O. Ingram [1987] *J. Bacteriol.* 169: 2591-2597; Brau, B., and H. Sahm [1986] *Arch. Microbiol.* 146: 105-110; Brau, B., and H. Sahm [1986] *Arch. Microbiol.* 144: 296-301; Neale, A.D., R.K. Scopes, R.E.H. Wettenhall, and N.J. Hoogenraad [1987] *Nucleic Acid. Res.* 15: 1753-1761; Ingram, L.O., and T. Conway [1988] *Appl. Environ. Microbiol.* 54: 397-404; Ingram, L.O, T. Conway, D.P. Clark, G.W. Sewell, and J.F. Preston [1987] *Appl. Environ. Microbiol.* 53: 2420-2425.

Molecular genetics offers the potential to combine in a single organism the pathway for anaerobic metabolism in pentose-utilizing enteric bacteria, such as *E. coli*, and the efficient pathway for ethanol production from an ethanol producer such as *Z. mobilis*. Thus, expression of the *Z. mobilis pdc* gene in enteric bacteria such as *E. coli*, *Erwinia chrysanthemi* and *Klebsiella planticola* partially diverts the flow of pyruvate to ethanol as a fermentation product by using low levels of native ADH

activity. More efficient ethanol production and higher concentrations of ethanol have been obtained with recombinant *E. coli* harboring the *Z. mobilis* genes encoding PDC and ADHII on a multi-copy plasmid. See Ingram et al. [1987], *supra*; Neale, A.D., R.K. Scopes, and J.M. Kelly [1988] *Appl. Microbiol. Biotechnol.* 29: 162-167. *E. coli* B (pLOI297) and *E. coli* ATCC 15224 (pLOI297) strains are superior constructs in terms of ethanol production and environmental hardiness. See Alterthum, F., and L.O.Ingram [1989] *Appl. Environ. Microbiol.* 55: 1943-1948. These recombinant *E. coli* efficiently ferment glucose, lactose, and xylose to ethanol.

The recombinant *E. coli* described above achieved useful levels of ethanol production using plasmid-borne ethanol-production genes from *Z. mobilis*. Further, initial testing of ethanol production in prototype strains was facilitated by placing the exogenous genes on a multi-copy plasmid. However, the exogenous genes were not completely stable because of the inherent instability of plasmids in the absence of selective pressure to ensure their retention in the host cell. Due to plasmid incompatibilities, moreover, the use of a typical *E. coli* expression plasmid for the ethanol-production genes precludes the most convenient means for introduction into a basic commercial ethanol- producer strain of additional exogenous genes for production of other selected products, such as valuable proteins.

Summary of the Invention

The present invention pertains to recombinant host cells that express chromosomally-integrated heterologous genes encoding useful polypeptides at high levels. According to one aspect of the present invention, such recombinant cells are obtained by first inserting a heterologous DNA segment encoding the desired polypeptide(s) into a host cell chromosome under the

control of an endogenous promoter which is preferably a strong promoter. Optionally, the transformed cells are then treated with a mutagen. Finally, transformants are tested for expression of heterologous genes, either by genetic selection or screening, to find those having a mutation that causes increased expression of the inserted DNA segment resulting in an increase in production of each polypeptide encoded by the inserted DNA segment.

The chromosomally-integrated, heterologous genes and the mutation that effects increased expression are extremely stable even in the absence of conditions that select for retention of increased gene expression. Also, the engineered hosts are environmentally safer than conventional, plasmid-based recombinant production systems because they do not carry mobile genetic elements.

Specifically exemplified is a recombinant enteric bacterium, *Escherichia coli*, that is capable of efficiently converting the entire range of biomass-derived sugars to ethanol using exogenous ethanol-production genes from an efficient ethanol producer, such as *Zymomonas mobilis*. These ethanol genes are stably integrated into the chromosome of the recombinant host under the control of an endogenous promoter for an *E. coli* pyruvate formate-lyase (*pfl*) gene. The chromosome of this host further comprises a mutation that increases production of the ethanol-production proteins according to this invention. These recombinant hosts can accommodate the usual *E. coli* expression plasmids for efficient co-production with ethanol of other desirable products, such as commercially valuable proteins. Accordingly, the residual biomass from high volume fermentation of the bacteria of this invention to produce ethanol may optionally provide one or more additional high-value products in great abundance.

More particularly, one aspect of the present invention relates to a recombinant host cell comprising a chromosome comprised of (a) a heterologous DNA segment

under transcriptional control of a promoter endogenous to that host cell, where the DNA segment encodes a desired polypeptide; and (b) a mutation that causes increased expression of the heterologous DNA segment resulting in an increased production by the recombinant host cell of the desired polypeptide, compared to production of that polypeptide by the recombinant cell in the absence of the mutation. This increased expression of the heterologous DNA segment is retained in the absence of conditions that select for cells having such increased expression. In one preferred embodiment the chromosome of the recombinant host cell comprises a heterologous DNA segment that encodes a plurality of genes. Advantageously, among the plurality of genes is a selectable marker gene.

The present invention also relates to a cell strain that is the product of a process comprising the steps of

(a) providing a culture comprised of host cells comprising a chromosome that encodes a promoter endogenous to these host cells and a host gene for integration of heterologous genes that is under transcriptional control of the endogenous promoter;

(b) transforming host cells in this culture with a heterologous DNA segment comprising

(i) a plurality of genes including a selectable marker gene and a gene encoding a desired polypeptide, and

(ii) sequences that are sufficiently homologous to the host gene and properly located in the heterologous DNA segment to enable integration into the host gene of the plurality of genes encoded by the heterologous DNA segment by means of homologous recombination;

(c) selecting for host cells in the above culture, or progeny thereof, that express the selectable marker gene at a first level;

(d) screening host cells selected in step (c), or progeny thereof, to obtain host cells that produce the desired polypeptide at an initial level;

(e) optionally exposing the host cells identified in step (d), or progeny thereof, to a mutagen under conditions such that mutations are created in the host cell chromosome; and then

(f) testing host cells produced in step (d) or step (e), or progeny thereof, for host cells that produce the desired protein at a level higher than the initial level, to obtain bacteria having a mutation that causes increased expression of the heterologous DNA segment.

This increased expression results in an increase in production by the host cells of the desired polypeptide compared to production of desired polypeptide by these host cells in the absence of the mutation. Furthermore, enhanced expression of the integrated heterologous DNA is retained in the absence of conditions that select for cells having such increased expression.

In preferred embodiments of the present invention, the above-described process is further qualified as follows:

(i) In step (b), the heterologous DNA segment further comprises a plasmid which comprises a replicon that is temperature-sensitive for replication.

(ii) In step (b), the process of transforming host cells also comprises introducing the heterologous DNA segment into host cells and growing these host cells under conditions that select for cells that express the selectable marker gene at the first level. The cells are grown at a temperature that does not permit replication of the plasmid, resulting in integration of the heterologous DNA segment into the host gene of the chromosome by a homologous recombination event.

(iii) In step (c), the process of selecting for host cells further comprises growing the host cells that express the selectable marker gene at the first level

under conditions that do not select for cells that express the selectable marker gene. In particular, these host cells are grown under these nonselective conditions at a first temperature that does permit replication of the plasmid, resulting in spontaneous excision from the host gene of the plasmid and the selectable marker gene by a second homologous recombination event. The host cells are then grown under the nonselective conditions at a second temperature that does not permit replication of the plasmid, resulting in host cells that retain the gene encoding the desired polypeptide in the absence of the selectable marker gene and the plasmid.

The basis of using nonselective conditions and a nonpermissive temperature for plasmid replication to obtain according to this aspect of the invention is that integration of the plasmid replicon into the chromosome provides a slight inhibition of growth rate. Therefore, strains which have excised plasmid grow slightly faster than those retaining integrated plasmid, thus providing the basis for enrichment of clones which have deleted the plasmid replicon and, coincidentally, the selectable marker gene.

In another preferred embodiment relating to this aspect of the invention, the above-described process is further qualified such that (i) in step (b) the heterologous DNA segment comprises a closed circular DNA lacking an ability to replicate and (ii) in step (f) the process of testing host cells comprises selecting for host cells produced in step (d) or step (e), or progeny thereof, that express the selectable marker gene at a second level that is higher than the first level, and then screening these host cells that express the selectable marker gene at the second level for host cells that produce the desired protein at a level higher than the initial level. When the selectable marker gene confers resistance to chloramphenicol, for example, host cells produced in step (d) or (e) of the above process may express the chloramphenicol resistance gene at a

first level that confers resistance to at least about 20 µg/ml of chloramphenicol. In those embodiments where the process involves further selection for cells carrying mutations that cause a second, higher level of expression of the chloramphenicol resistance gene, the second level of expression may confer resistance to at least about 100 µg/ml of chloramphenicol. Advantageously, selection for mutant cells with a higher level of expression of chloramphenicol resistance may be carried out using about 600 µg/ml of chloramphenicol.

A recombinant host cell of this invention can be a microbial cell, such as an enteric bacterium. Illustrative of suitable enteric bacteria in this regard are strains of *Erwinia chrysanthemi*, *Escherichia coli* and *Klebsiella pneumoniae*.

The heterologous DNA segment which is chromosomally integrated into the genome of the recombinant host, in accordance with the present invention, is preferably under the control of a strong endogenous promoter, for example, the pyruvate formate-lyase (*pfl*) promoter.

In certain embodiments, the heterologous DNA segment encodes an alcohol dehydrogenase and a pyruvate decarboxylase from an organism that produces high levels of ethanol. Such enzymes are exemplified by an alcohol dehydrogenase and a pyruvate decarboxylase encoded by genes from *Zymomonas mobilis*. In a preferred embodiment of the present invention, the recombinant host cell is able to produce ethanol by fermentation, for example, of glucose or xylose, with theoretical yields corresponding to conversion of at least about 90% or 100%, respectively, of added sugar to ethanol. In some cases the observed yields of ethanol appear to exceed those possible based on the amount of added sugar, a result reflecting co-catabolism of complex nutrients to pyruvate and thus to ethanol.

In accordance with an additional aspect of the present invention, a recombinant host cell as described above is provided that contains a chromosome comprising

a further mutation, particularly a mutation in a fumarate reductase (*frd*) gene, that impairs succinate production, reducing production of acid which can inhibit ethanol production.

A recombinant host cell within the present invention optionally may comprise a further mutation that impairs recombination in the cell, to make the cell safer environmentally by reducing its ability to interact with mobile genetic elements. In one embodiment, the mutation that impairs recombination comprises a mutation in a *recA* gene.

A recombinant host cell of this invention can be produced by various processes in addition to those particularly exemplified here. Exemplary of such processes are genetic-engineering methods for insertion of a heterologous DNA segment into a predetermined location on a host cell chromosome, and for generation and identification of mutations which enhance expression. In particular, standard molecular genetic analyses of a recombinant microbial strain of the present invention will enable one to employ other methods to make recombinant host cells which likewise fall within the present invention. For example, knowledge of the DNA-sequence changes associated with a particular mutation effected in accordance with the present invention enables the design, synthesis and insertion into a host chromosome of a heterologous DNA segment containing not only genes coding for desired proteins but also a promoter and a mutation effecting high-level expression of the genes. Such a genetic construct, comprising a heterologous DNA segment that includes an appropriate mutation and promoter with the desired genes, is therefore another embodiment within the present invention.

Brief Description of the Drawings

Figure 1 is a schematic diagram illustrating construction of an integration vector (pLOI543), containing a temperature-conditional replicon (pSC101; Hamilton, C. M., M. Aldea, B. K. Washburn, P. Babitzke, and S. R. Kushner [1989] *J. Bacteriol.* 171: 4617-22) for the integration of ethanol-production genes (*Z. mobilis pdc* and *adhB* genes) and a chloramphenicol resistance gene into a *pfl* gene of an *E. coli* chromosome. Abbreviation: Klenow, convert to blunt by filling bases in overhang region using the Klenow fragment of *E. coli* DNA polymerase I.

Figure 2 is a schematic diagram illustrating construction of a plasmid (pLOI510) for the integration of *Z. mobilis pdc* and *adhB* genes into a *pfl* gene of an *E. coli* chromosome using a vectorless, circularized DNA fragment. Abbreviations: Klenow, convert to blunt by filling bases in the overhang region using the Klenow fragment of DNA polymerase I; Cir. Frag., circularized SalI fragment from pLOI510 which has been eluted from an agarose gel and ligated to form closed circles.

Figure 3 illustrates ethanol (A, C, E, G) production and growth (B, D, F) during batch fermentation. A and B. Fermentation of 10% glucose. Symbols: ●, plasmid-based ethanol production strain ATCC11303(pLOI297); ○, chromosomally-integrated ethanol production strain lacking an expression enhancing mutation, KO2. C. and D. Fermentation of 10% glucose. Symbols: ○, a chromosomally-integrated strain lacking a spontaneous expression enhancing mutation, KO3; ●, chromosomally-integrated strain containing a spontaneous expression enhancing mutation, KO4; ▲, KO4 supplemented with 22 mM sodium acetate. E. and F. Fermentation of 8% xylose. Symbols: ●, KO4; ▲, KO11, a further mutant of KO4, lacking fumarate reductase activity (*frd*); □, KO12, a further mutant of KO11, carrying a *recA* mutation. G. Fermentation by KO20, a chromosomally-integrated strain

with an induced mutation. Symbols: ■, 10% glucose, ●, 8% xylose.

Figure 4 illustrates fermentation of 10% glucose by KO11 (*frd*) without pH control. A. Growth (●) and ethanol production (○). B. pH of broth (▲).

Detailed Description of Preferred Embodiments

The present invention relates to a recombinant host cell comprising a chromosome comprised of (a) a heterologous DNA segment under transcriptional control of a promoter endogenous to that microbial cell, where the DNA segment encodes a desired polypeptide; and (b) a mutation that causes increased expression of the heterologous DNA segment resulting in an increase in production by the host cell of the desired polypeptide compared to production of that polypeptide by the host cell in the absence of this mutation. Enhanced expression of the heterologous DNA segment is retained in the absence of conditions that select for cells having such increased expression.

Definitions: In the present context, "heterologous" DNA segment means that the DNA segment contains a sequence that is different from the sequence in the corresponding position downstream from the endogenous promoter in the chromosome of the cell into which the heterologous segment has been inserted. Thus, the heterologous DNA segment of the present invention includes a DNA segment taken from one location in a host chromosome and inserted into another location (under control of an endogenous promoter other than the one naturally associated with that segment), as well as a DNA segment from another organism. The nucleotide sequence of the heterologous DNA segment encodes one or more structural genes and is derived from any genetic source, including, for example, eukaryotic or prokaryotic cellular or viral genomes, or from artificial coding sequences created by genetic engineering.

The heterologous segment is under the transcriptional control of an endogenous promoter by virtue of being integrated into a host cell chromosome on the downstream (5') side of the promoter. As previously noted, the endogenous promoter is preferably a "strong" promoter, in the sense that it provides a high level of gene expression in relation to a more typical microbial promoter, such as the lactose operon (*lac*) promoter of *E. coli*. Among strong promoters are those that include a single site for promoter activity, i.e., for binding RNA polymerase and directing the enzyme to the correct transcriptional start site, and those that include a plurality of such sites. Illustrative of the former (single-site) category of strong promoters is the well-known tryptophan (*trp*) promoter. The latter (multi-site) category is exemplified by the promoter for a pyruvate formate-lyase (*pfl*) gene, homologous variants of which are found in *E. coli* and other enteric bacteria. Pyruvate formate-lyase is normally expressed at a high level, particularly under the anaerobic conditions which pertain during fermentation without forced aeration. The *pfl* promoter in fact includes seven sites for promoter activity, as reflected, for example, in sequence analyses of the *pfl* mRNA and in "footprinting" data indicating where RNA polymerase is bound, or in "primer extension" analyses indicating the 5' end of a mRNA.

In this description, "mutation" denotes a relatively permanent change in hereditary material, typically involving a biochemical change in the codons that make up genes but also possibly involving a physical change in chromosome relations. A mutation suitably employed according to the present invention causes increased expression of the heterologous DNA segment, resulting in enhanced production by the host cell of each polypeptide encoded by that segment, relative to production of each polypeptide by the host cell in the absence of the mutation. The increased expression thus achieved is retained in the absence of conditions that select for

cells having such increased expression. For example, in bacterial strains having mutations, according to the present invention, that increase expression of both antibiotic resistance and ethanol-production enzymes integrated into a chromosome, no loss of high-level expression of the integrated genes is detectable after as many as 68 population doublings, even without selection with antibiotic.

Transformed host cells bearing a mutation according to the present invention may be detected conveniently by means of the increased expression of one or more polypeptides encoded by the inserted DNA segment, according to standard genetic methodology. Increased expression of a polypeptide may be determined by direct detection of a polypeptide or inferred from an activity attributable to that polypeptide. Advantageously, one of the genes encoded by the inserted DNA segment can be a selectable marker gene, for example, a gene which confers antibiotic resistance to the host. In this case, mutant cells with increased expression of the antibiotic resistance gene may be selected conveniently using antibiotic concentrations above a first level that inhibits host cells lacking a mutation that increases expression of the heterologous DNA segment.

More particularly, it has been discovered that mutations suitable for use in the present invention can be obtained reliably as spontaneous mutants or by induction via conventional mutagenesis, followed by selection or by screening for cells having the desired phenotype of high-level expression of integrated genes. Thus, suitable mutations in certain bacteria are found to arise spontaneously at a frequency on the order of 10^{-4} to 10^{-5} upon selection for cells having increased expression of an integrated chloramphenicol resistance gene, such that cell growth occurs at a concentration of antibiotic 30 times higher than that tolerated in the absence of such a mutation. Similarly, after treatment of certain bacterial cells with a mutagen, suitable mutations can be

obtained at a frequency of about 0.5 - 1 x 10^{-4} of the survivors of mutagenesis by screening for cells having expression of ethanol-related enzymes at levels about ten times higher than in the absence of such mutagenic treatments.

Mutations suitable for use in the present invention actually cause overexpression of inserted genes to the extent that polypeptide production from a single copy of a gene chromosomally inserted, according to this invention, is comparable to production achieved with multiple copies of the same gene carried on a multi-copy plasmid. For example, bacterial cells carrying such a suitable mutation can express integrated genes, say, for chloramphenicol resistance and ethanol production, at levels that are functionally equivalent to a cell containing 30 to 300 copies of the same genes on a multi-copy plasmid.

A molecular basis has not been determined for mutations identified, as described herein, for use according to the present invention. Nevertheless, the magnitude of the increase in gene expression (on the order of about ten fold, for example) that is obtained in a single screening or selection step is substantially greater than that expected for a mutation mechanism involving gene duplication. To achieve a comparable increase in selectable gene expression by means of gene duplication typically requires repeated selection steps with progressively higher levels of selective agent. Moreover, the low (in fact, undetectable) frequency of reversion of the present mutations also militates against gene duplication as a basis for these mutations. In fact, it is known that mutations which produce increased gene expression by means of tandem gene repetition are typically unstable and cannot be retained in the absence of conditions that select for cells having such increased gene expression, in contrast to mutations of the present invention. Conversely, the observed frequency of suitable mutations arising spontaneously,

according to the present invention, is compatible with a causal mechanism involving point mutations.

Selection of mutations that enhance expression of integrated genes using a selectable marker gene may be performed pursuant to the present invention in any type of host cell for which practical genetic methods are known in the art for selecting clones having mutations that are inducible at a frequency on the order of at least about 10^{-4} per survivor of mutagenic treatment. Similarly, screening for mutant host cells pursuant to the present invention requires practical methods for preparation of sufficient numbers of clones, each comprising enough cells for detection of high-level expression of a desired integrated gene by a method appropriate to the particular desired gene.

Such selection or screening for suitable mutations is most easily effected with host cells which are readily culturable in large quantities, as in the case of prokaryotic (bacterial) and yeast cells. For purposes of this description, such a host is denoted a "microbial cell," and the strain it comprised is a "microbial strain." Under this rubric of microbial cells, enteric bacteria like *Erwinia chrysanthemi*, *Escherichia coli* and *Klebsiella planticola* are particularly attractive hosts because they are capable of utilizing a wide variety of sugars, including pentoses and lactose. While microbial cells are preferred hosts,

the present invention also contemplates the use of other types of cellular hosts, including fungal cells and eukaryotic (animal, insect and plant) cells, into which a heterologous DNA segment can be inserted under control of an endogenous promoter. Thus, the host cell need only be amenable to a selection or screening regimen for cells having increased expression of the integrated heterologous DNA, as described above, thereby to obtain suitable enhanced-expression mutants.

Embodiment relating to ethanologenic *E. coli*: An illustrative application of the present invention

involves the integration of *Z. mobilis* genes for ethanol production into the *pfl* region of an *E. coli* chromosome. The *pfl* gene is central to normal fermentative metabolism, catalyzing the conversion of pyruvate to formate plus acetyl-CoA and providing an essential source of acetyl units for biosynthesis. Insertional inactivation of this gene thus represents inhibition of a competing branch point for the diversion of pyruvate away from production of ethanol by the microorganism. Additional genetic improvements are also described which eliminate succinate production and inactivate the *recA* gene.

These recombinant bacteria are useful for the manufacture of recombinant polypeptides as co-products during ethanologenic fermentation of biomass sugars. As a result of integration into the chromosome, the stability of the ethanol production trait is improved significantly compared to comparable plasmid-based systems for the purposes of industrial-scale fermentation to produce ethanol: 100% retention of the ethanol genes after 68 generations compared to only 97% with a comparable plasmid-based construct. Also, elimination of the plasmid carrying ethanol-production genes allows insertion of plasmids for producing co-products without interference with the ethanol production ability.

Biological deposits: The following cultures have been deposited with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland, 20852, USA. Table 1 lists the accession numbers assigned to the cultures by the repository.

Table 1. Biological deposits

Culture	Accession Number
E. coli (pLOI510)	ATCC 68484
E. coli (pLOI543)	ATCC 68485
E. coli KO4	ATCC 55123
E. coli KO11	ATCC 55124
E. coli KO12	ATCC 55125
E. coli KO20	ATCC 55126

The subject cultures have been deposited under conditions that assure that access to the cultures will be available during the pendency of this patent application to one determined by the Commissioner of Patents and Trademarks to be entitled thereto under 37 CFR §1.14 and 35 USC §122. The deposits are available as required by foreign patent laws in countries wherein counterparts of the subject application, or its progeny, are filed. It should be understood, however, that the availability of the deposits does not constitute a license to practice the subject invention in derogation of patent rights granted by governmental action.

Further, the subject culture deposits will be stored and made available to the public in accord with the provisions of the Budapest Treaty for the Deposit of Microorganisms, i.e., they will be stored with all the care necessary to keep them viable and uncontaminated for a period of at least five years after the most recent request for the furnishing of a sample of the deposit(s), and in any case, for a period of at least 30 (thirty) years after the date of deposit or for the enforceable life of any patent which may issue disclosing the cultures. The depositor acknowledges the duty to replace the deposits, should the depository be unable to furnish a sample when requested, due to the condition of the deposit. All restrictions on the availability to the public of the subject culture deposits will be irrevocably removed upon the granting of a patent disclosing them.

Integration of *Z. mobilis pdc* and *adhB* genes into the

E. coli B chromosome: Two approaches embodied in examples set out below represent different strategies which can be employed, in accordance with the present invention, to construct a bacterial strain, such as *E. coli* (ATCC 11303), in which a foreign gene (here, the *Z. mobilis* genes for ethanol) is integrated into a chromosomal gene, illustrated by the *pfl* gene. The first approach entails the use of a derivative of the temperature-conditional integration vector developed by Hamilton et al. See Hamilton, C. M., M. Aldea, B. K. Washburn, P. Babitzke, and S. R. Kushner [1989] *J. Bacteriol.* 171: 4617-22; and Example 3, *infra*.

Thus, as shown in the examples, ATCC 11303 was transformed with a temperature-dependent replication plasmid, pLOI543, followed by temperature-based selection and enrichment for two homologous recombination events, 64.5% of the colonies were sensitive to chloramphenicol (Cm), indicating loss of plasmid. Of these Cm-sensitive clones, 5.9% formed pink colonies on aldehyde test plates indicating the presence of *Z. mobilis* ADHII. Two of these clones having retained integrated ethanol-production genes but having lost the Cm-resistance gene and plasmid were selected for further study and designated KO1 and KO2.

Via a second approach, reflected in Example 4, a Cm-resistant transformant was obtained using the circularized SalI fragment from plasmid pLOI510 and was designated KO3. In this approach no replicating plasmid is introduced into the cell; only the ethanol-production genes and associated Cm-resistance gene are integrated into the chromosome. The KO3 clone also formed pink colonies identical to KO1 and KO2 on aldehyde indicator plates. Control ATCC 11303 formed white colonies (negative), while ATCC 11303 (pLOI297) carrying *Z. mobilis* ethanol genes on a plasmid formed intensely red colonies indicating a high level of *Z. mobilis* gene expression.

Subsequently, isolation of vector from these three

recombinants was attempted by the alkaline SDS lysis method. No vector was visible in agarose gels of these preparations which had been stained with ethidium bromide. These preparations were also tested in transformation experiments with TC4 as the host with selection for Cm resistance. No transformants were recovered further confirming the absence of vector.

Fermentation by strains KO1, KO2, and KO3: Figure 3 A and B shows a comparison of fermentations by strains KO2 and ATCC 11303 (pLOI297) with 10% glucose. Fermentation by strain KO1 was identical to that by KO2 and is not presented. These new constructs were very inefficient producers of ethanol as indicated by the low volumetric productivity and ethanol yield (Table 2). Growth was limited to less than half that of ATCC 11303 (pLOI297) and only 4 g/liter of ethanol was produced after 72 hours. Although strain KO3 was slightly better than KO1 and KO2, it remained a very poor producer of ethanol (Figure 3 C-D; Table 2).

Table 2. Ethanol production from glucose and xylose by recombinant strains of *E. coli* (ATCC11303)

Parameter[a]	10% Glucose					
	pLOI297b	KO1	KO2	KO3	KO4	KO5
Base (mmoles/g sugar)	1.1	5.7	6.3	5.5	1.3	1.4
Ethanol Yield (g/liter)	48.8	4.0	4.0	10.4	52.8	52.8
Ethanol Yield (g/g sugar)	0.52	0.05	0.05	0.13	0.56	0.56
Theoretical Yield (%)	101	10	10	26	110	110
Vol. Prod.[c] (g/liter*h)	1.9	0.3	0.3	0.4	1.5	1.5
30-h Ethanol (g/liter)	41.8	3.2	3.2	6.4	36.0	36.0
Cell Yield (g/g sugar)	.048	.021	.021	.028	0.44	.040

[a] Calculations based on total sugar initially added.

[b] ATCC11303(pLOI297) has plasmid-borne ethanol genes.

[c] Vol. Prod., volumetric Productivity = yield/time

Table 2. continued

Parameter	10% Glucose				
	KO10 (recA)	KO11 (frd)	KO11 (frd)[a]	KO12 (frd,recA)	KO4 +Ac[a]
Base (mmoles/g sugar)	1.1	0.6	0	1.1	1.0
Ethanol Yield (g/liter)	51.2	52.8	38.8	54.4	54.4
Ethanol Yield (g/g sugar)	0.54	0.54	0.39	0.57	0.57
Theoretical Yield (%)	107	107	76	112	112
Vol. Prod. (g/liter*h)	1.8	1.7	1.4	1.2	1.6
30-h Ethanol (g/liter)	38.0	38.0	29.0	30.4	38.5
Cell Yield (g/g sugar)	.041	.042	.041	.035	.045

[a] Fermentation conducted without pH control.

[b] Supplemented with 3 g/liter sodium acetate (22 mM final concentration).

Table 2. continued

Parameter[a]	8% Xylose			
	pLOI297[b]	KO4	KO11(*frd*)	KO12(*frd*,*recA*)
Base (mmoles/g sugar)	1.4	1.6	0.5	0.64
Ethanol Yield (g/liter)	36.0	36.0	41.6	40.8
Ethanol Yield (g/g sugar)	0.47	0.47	0.53	0.53
Theoretical Yield (%)	94	94	104	103
Vol. Prod. (g/liter*h)	1.0	1.1	1.3	1.1
30-h Ethanol (g/liter)	30.0	25.6	30.4	26.0
Cell Yield (g/g sugar)	.050	.047	.051	.048

[a] Calculations based on total sugar initially added.

[b] ATCC11303(pLOI297) has plasmid-borne ethanol genes.

Large volumes of base were consumed for maintenance of pH during fermentations by these three new strains, indicating excessive production of acidic fermentation products.

Enhancement of expression of integrated *pdc* and *adhB* genes by mutations: The pink phenotype observed on aldehyde indicator plates appeared to indicate insufficient expression of *Z. mobilis* genes for ethanol production. A mutation that causes increased expression of the integrated genes in KO2 was obtained by screening for dark red phenotype on aldehyde indicator plates after mutagenesis with ethyl methane sulfonate under standard conditions well known in the art. Approximately 200 plates were analyzed with 200 to 400 colonies per plate. Four dark red clones were isolated, the increased color being indicative of increased expression of ADHII. One of these was designated strain KO20.

Selection for resistance to high levels of Cm was used to enrich for spontaneous mutants of KO3 to determine whether mutants expressing antibiotic resistance at a higher level also expressed the other inserted genes at a higher level. Serial dilutions of an overnight culture were plated on Luria agar plates containing 2% glucose and 600 μg/ml of Cm. Large raised colonies which are indicative of high-level expression of *Z. mobilis pdc* and *adhBita* genes were observed after overnight incubation at a frequency of approximately 1 per 100,000 plated cells. All of these colonies exhibited a dark red phenotype on aldehyde indicator plates identical to ATCC 11303 (pLOI297), the plasmid-based construct which is an excellent ethanol producing strain. Two mutants were retained for further study, strains KO4 and KO5. The lack of vector in these strains was again confirmed by the failure of DNA preparations from KO4 and KO5 to transform TC4 during selection for Cm resistance and by the absence of vector DNA in agarose gels stained with ethidium bromide.

Fermentation by strains KO4, KO5 and KO20:

Figure 3 C and D illustrate the fermentation of 10% glucose by KO4 and KO5, two strains in which spontaneous mutations that enhance expression of integrated ethanol-production genes were co-selected by selection for resistance to high levels of an antibiotic. Both strains were identical and only fermentation by KO4 has been plotted. Growth, cell yield, and ethanol yield by these improved constructs were almost equivalent to those of the plasmid-based construct, ATCC 11303 (pLOI297). See Table 2. Although the rate of ethanol production as indicated by volumetric productivities from the early stages and by the level of ethanol achieved after 30 hours was somewhat slower than ATCC 11303 (pLOI297), theoretical yields with KO4 and KO5 were higher and exceeded 100% based on added glucose. This higher yield during slower fermentation reflects co-catabolism of complex nutrients to pyruvate and thus to ethanol. These complex nutrients serve as the primary nitrogen source for biosynthesis. Continued denitrification caused an increase in the pH with KO4, KO5 and ATCC 11303 (pLOI297) after sugars were exhausted. The resulting rise in pH provides a convenient method to monitor sugar exhaustion.

The fermentation of 8% xylose by strain KO4 and ATCC 11303 (pLOI297) were also compared (Fig. 3 E and F). KO4 was equivalent to the plasmid-based strain in ethanol yield, although the rate of ethanol production was slightly slower as evidenced by the ethanol level after 30 hours (Table 2).

Figure 3 G illustrates the fermentation of 10% glucose and 8% xylose by strain KO20 which comprises an induced mutation that enhances expression of integrated ethanol-production genes. Fermentation of both sugars by this strain carrying an induced mutation was essentially the same as for strains KO4 and KO5 which carry spontaneous mutations selected by high levels of antibiotics.

Effect of added acetate: During anaerobic growth, the *pfl* gene product has been shown to be the primary route for acetate production and the primary source of acetyl-CoA for biosynthesis (7,17). Insertional inactivation by the *Z. mobilis* ethanol-pathway genes may lead to an acetate deficiency for lipid synthesis in derivatives of ATCC 11303. Supplementation with sodium acetate was found to improve the rate of ethanol production with a small increase in yield (Fig. 3 C and D; Table 2).

Expression of *Z. mobilis* enzymes in recombinant *E. coli*: The specific activity of *Z. mobilis* PDC was measured in French press extracts of selected recombinant strains. PDC is a relatively thermostable enzyme and activities were measured in extracts after heat inactivation of native *E. coli* activities which complicate such measurements. No activity was detected in the control, strain ATCC 11303. Both KO2 and KO3 produced low levels of activity, 0.2 U/mg protein. Ten-fold higher activity was present in extracts of KO4 (2.1 U/mg protein), the high Cm-resistant mutant. The level of PDC in KO4 was almost equivalent to the level produced in the plasmid-based construct, ATCC 11303 (pLOI297), and similar to that found in native *Z. mobilis* (Ingram, L.O., and T. Conway [1988] *Appl. Environ. Microbiol.* 54: 397-404).

Protein extracts from these strains were also examined by SDS-PAGE. Numerous changes in proteins were observed between ATCC 11303 and recombinant derivatives in addition those attributable to *Z. mobilis* genes. KO4 and ATCC 11303 (pLOI297) contained higher levels of a protein band in the 60,000 MW region corresponding to the size of PDC than were present in KO2 and KO3. This band was absent in the ATCC 11303 control. Many endogenous proteins were found in the 38,000 MW region where *Z. mobilis* ADHII would be found, obscuring differences in expression.

Organic acid production by *E. coli* ATCC11303 constructs: Considerable amounts of base were consumed by ethanologenic strains of *E. coli* during fermentations (Table 2) indicating the production of acids as co-products. As shown in Table 3, high levels of three acids are produced by the ATCC 11303 parent and by KO3. Although ATCC 11303 (pLOI297)

Table 3. Production of acidic fermentation products[a].

Recombinant[b]	Organic Acid (mM)		
	Acetic acid	Lactic acid	Succinic acid
ATCC11303 (*None*)	66	641	58
ATCC11303 (pLOI297) (plasmid-borne)	21	32	49
KO1 (integrated, no mutation)	18	673	62
KO2 (integrated, no mutation)	18	602	57
KO3 (integrated, spontaneous)	69	525	66
KO4 (integrated, spontaneous)	22	29	70
KO10 (KO4, *recA*)	18	20	73
KO11 (KO4, *frd*)	14	32	2
KO12 (KO4, *recA*, *frd*)	6	40	2
KO20 (integrated, induced)	4	60	2

[a] Average of two fermentations with 10% glucose, sampled after 72 h.

[b] Notes in parentheses indicate status of exogenous ethanol genes and related mutations that enhance ethanol production.

and KO4 produced lower levels of acetic and lactic acids, succinate production by KO4 remained high. To eliminate succinate production, a *frd* mutation was introduced by insertion of a Tn10 vector using standard genetic methods, including selection for a tetracycline resistance marker of Tn10, followed by elimination of Tn10 using fusaric acid selection, resulting in an *frd* deletion due to imprecise excision of Tn10. The resulting strain (KO11) lacked fumarate reductase activity.

This *frd* mutation reduced the level of succinate produced during glucose fermentation to 3% that of the parent, KO4 (Table 3). However, without titration of acid during fermentation, this low level of acid production was still sufficient to reduce the pH of fermentations run without titration and, therefore, to reduce ethanol productivity from glucose (Fig. 4, Table 2).

However, with acid titration, the elimination of fumarate reductase did improve the rate of ethanol production, cell yield, and ethanol yield from xylose (Table 2). With titration, volumetric productivity from glucose was also increased by this mutation, although ethanol yield remained essentially the same (Table 2).

Comparison of the stability of chromosomally-integrated and plasmid-based genes in *E. coli*. ATCC 11303 (pLOI297), KO4 and KO5 were grown in the Luria broth containing 10% (w/v) glucose without antibiotic selection for up to 68 generations at 30oC. Cultures were plated on selective and nonselective plates and on aldehyde indicator plates after 48 and 120 hours to determine the ratio of Cm-resistant CFU to total CFU as well as the proportion of ethanol-producing colonies. Table 4 shows that the inserted genes of KO4 and KO5 were stably maintained as a chromosomal insertion.

Table 4. Stability of chromosomally-integrated genes compared to plasmid-based genes in *E. coli* B[a]

	Recombinant Percentage retaining traits production (number of generations)	
KO4	100 (38.5)	100 (68.5)
KO5	100 (38.7)	100 (68.7)
Tc *E. coli* B (pLOI297)	100 (37.3)	97 (67.3)

[a]Transformed *E. coli* B were grown at 30°C in Luria broth containing 10% (w/v) glucose without antibiotic selection. Tc resistance and ethanol genes were coordinately lost from the plasmid-based construct.

Strains KO4 and KO5 in which the genes for ethanol production have been integrated into the chromosome are superior to the prior construct with plasmid pLOI297, see Alterthun, F., and L.O. Ingram [1989] *Appl. Environ. Microbiol.* 55: 1943-1948, in terms of retention of the recombinant trait, ethanol production. Even when the rate of plasmid loss is low, as in *E. coli* strains carrying pLOI297, strains carrying integrated exogenous genes according to the present invention offer considerable advantages over comparable plasmid-based strains for the purpose of commercial ethanol production which involves the scaling up of small cultures to millions of gallons of fermentation broth.

Effect of *recA* mutation: A *recA* mutation was also introduced into KO4 and KO11 so that these strains could be used as hosts for recombinant plasmids. The resulting strains were designated KO10 and KO12, respectively. The *recA* mutation did not affect the production of acetic, lactic, or succinic acids but did reduce growth and slow fermentation (Table 2) in the *recA*, *frd* mutant

(KO12). The basis of this effect is unknown, and it may represent secondary mutations which have been created during construction.

Genes that can be chromosomally inserted according to the present invention: The present invention can be practiced using genes other than the specific *adh* and *pdc* genes exemplified herein. It is now well established that the enzymes of glycolysis exhibit a great deal of conservation of primary sequences. See, for example, Conway, Sewell, and Ingram [1987] *J. Bacteriol.* 169: 5653-5662. This high level of conservation enables those skilled in the art to isolate functionally equivalent, genetically related enzymes from other organisms using primary information from one or more members of an enzyme family. Indeed, just such an approach has been used successfully to clone the pyruvate decarboxylase gene from maize using the current inventors' information on the *Z. mobilis pdc* and the *pdc* of *S. cerevisiae* to design a DNA probe. See Kelly, P.M. [1989] *Plant Molecular Biology* 13: 213-222. Alternative strategies using entire genes as probes can also be used. Thus, for purposes of this invention, it does not matter if the pyruvate decarboxylase activity is provided by a gene from *Z. mobilis*, as exemplified in the subject invention, or from genes which specify the same enzymatic activity from corn (which has been cloned and sequenced), yeasts or another organism. Also, in order to practice the invention, it does not matter if the alcohol dehydrogenase activity is provided from a gene from a horse, yeast, human or insect, or from another bacterial gene. Since expression of alcohol dehydrogenase activity can be observed directly on aldehyde indicator plates, sequence information would not necessarily be the best approach to the isolation of additional genes encoding proteins which exhibit this enzymatic activity. However,

whether or not sequences are to be used for such isolation is not really critical. Indeed, many alcohol dehydrogenase genes are already in hand and well described in many papers.

Z. mobilis contains two genes encoding functional alcohol dehydrogenase genes. The one which has been exemplified here, *adhB*, is evolutionarily related to a butanol (alcohol) dehydrogenase from *Clostridium acetobutylicum*, propanediol (alcohol) oxidoreductase from *E. coli*, and ADHIV alcohol dehydrogenase from *Saccharomyces*. All have been clones and sequenced. The second *Z. mobilis* gene encoding alcohol dehydrogenase, *adhA*, is a zinc alcohol dehydrogenase and has recently been cloned and sequenced by us. This *adhA* is evolutionarily related to the typical alcohol dehydrogenases described in animals, plants, and the dominant gene in yeasts based upon comparisons of primary structure deduced from nucleotide sequences which are available for all. We have found that this *adhA* gene substitutes quite nicely for the original *adhB* gene, as expected pursuant to the present invention.

The synthesis of a protein with pyruvate decarboxylase activity (pyruvate converted to acetaldehyde plus carbon dioxide) can be observed directly on aldehyde indicator plates. The expression of alcohol dehydrogenase activity can also be directly observed on aldehyde indicator plates. Therefore, sequence information would not necessarily be the best approach to locating other *adh* or *pdc* genes, although, as described above, sequence information from our work was used recently to isolate the corn *pdc* gene. Thus, many other *pdc* and *adh* genes which provide a functional equivalent can be isolated from other organisms. It is entirely predictable that these other genes would be suitable replacements for the *Z. mobilis pdc* and *adh*

genes available for use, and other such genes can be identified either by use of the current genes as probes or, more preferably, by observing activity on indicator plates.

More generally, many genes other than ethanol-production genes could be incorporated into a chromosome and expressed according to the present invention. These include essentially any desired polypeptide that can be expressed in a recombinant host, for example, are genes encoding insulin, growth hormones, and commercially important enzymes.

Utility of exemplary bacteria: Certain bacteria and other simple organisms are capable of actively metabolizing a wide variety of substrates, including hexoses, pentoses, and lactose. This characteristic makes *E. coli* an attractive host for recombinant DNA production methods. The invention described here permits the use of recombinant bacterial strains for the economical production of ethanol from a variety of biomass sources, particularly from under-utilized sources of biomass such as hemicellulose (comprising xylose, arabinose, and other sugars), which represents a major portion of wood and inedible plant parts, and whey (lactose). Also, organisms with special capabilities, such as production of extracellular enzymes for the degradation of complex polymers, can be converted to ethanol producers according to the present invention.

Thus, one aspect of the present invention provides an improved organism for the production of ethanol. An *E. coli* cell has been transformed such that *Z. mobilis* genes coding for *adh* and *pdc* have been incorporated into the host chromosome. In the past, ethanol-production capability has been conferred on *E. coli* by transformation with a plasmid comprising the two genes needed for ethanol production. The success of these previous

transformations resulted from the very high levels of the ethanol-producing enzymes which were made from multiple copies of the genes, typically, 30 to 300 copies per cell.

The initial constructs described here in which single copies of *pdc* and *adh* genes were integrated into the host chromosome provided insufficient levels of PDC and ADH to divert metabolism to ethanol and duplicate the level of functionality provided by multiple copies of these genes on a plasmid. Subsequently, spontaneous or induced mutations described above produced cells which simultaneously increased expression of all chromosomally-integrated genes, including that for chloramphenicol resistance, when present, and those for ADH and PDC. These mutants produce high levels of ethanol-production enzymes and are thus functionally equivalent to previous bacteria containing multi-copy plasmids. In the case of spontaneous mutations in a strain carrying a Cm-resistance marker gene as well as ethanol genes, the result whereby increased expression of the Cm-resistance gene also indicated increased expression of the ethanol-related enzymes is particularly surprising because the Cm-resistance gene can include its own promoter and can even be downstream from a transcription terminator of an *adhB* gene.

Another aspect of this invention concerns the use of the recombinant ethanol-producing bacteria for the efficient production of recombinant proteins; that is, the recombinant cells can be further transformed with genes coding for useful proteins. These additional genes can also be incorporated into a chromosome, or they can be plasmid-borne. Indeed, since high-level expression of the integrated (in this example, ethanol-production) genes, in accordance with the present invention, obviates the need for a plasmid to carry

them, recombinant cells of the present invention are particularly well-suited to receive and maintain additional genes carried on a plasmid. An additional mutation, such as a *recA* mutation, that inhibits homologous recombination will enhance the environmental safety of such hosts in the context of recombinant protein production.

It should be noted that the accumulation of organic acids from sugar metabolism is generally regarded as a consequence of fermentation during anaerobic growth. But appreciable quantities of acetate are generally produced by *E. coli* even during rapid agitation under aerobic conditions. The production of acetate is progressive from the earliest stages of growth and is not limited to the later stages, when cell density is high. This acid production from glucose even under aerobic conditions serves to limit growth in broth and on solid medium, as demonstrated by the increased final cell density in medium supplemented with phosphate buffer.

The conversion of a host organism to ethanolic fermentation can be used to enhance the production of a variety of recombinant products. The maintenance of function in these products is related to the pH of the broth during growth in dense culture. The extent of this acidification per unit of cell protein is minimized by the production of ethanol rather than of organic acids. Oxygen transfer is frequently a major limitation during the growth of dense cultures of microorganisms, and it is this limitation which results in acid production and pH drift of the growth medium. In recombinants producing ethanol as a fermentation product, the ethanologenic enzymes divert part of the pyruvate from glycolysis to acetaldehyde and reoxidize NADH to produce ethanol, a less damaging product of metabolism. Strains containing both functional

respiratory chains for oxidative phosphorylation and ethanol-production enzymes can be grown to even higher cell densities because of the operation of both systems during the regeneration of NAD^+ and a reduction in acidic waste products. Such inherent flexibility results in less stringent process-control requirements, as well as increased yields of recombinant products.

The ethanol-producing bacterial strains of this invention are thus superior hosts for production of recombinant proteins under anaerobic conditions with minimal acid production. Many recombinant proteins contain cysteine or disulfide bridges, and proper folding or reactions of these is an essential feature to form the active enzyme. Since formation of disulfide bonds is promoted by oxygen, synthesis of such proteins under anaerobic conditions provides less opportunity for improper folding prior to isolation and folding under controlled conditions, potentially resulting greater recovery of biologically active product.

From the foregoing it should be readily apparent to one skilled in the art that the ability conferred by the present invention, to transform genes coding for a protein or an entire metabolic pathway onto a chromosome, is extremely useful. Envisioned in this regard, for example, is the application of the present invention to a variety of situations where genes from different genetic loci are placed on a chromosome. The placement of genes coding for ethanol production is only one example of this novel inventive concept. Pursuant to the principles described here, genes coding for alcohol dehydrogenase activity from a variety of organisms can be combined with genes coding for the pyruvate decarboxylase activity from a variety of organisms in order to create the desired pathway. Genes coding for proteins needed for other pathways could

also be incorporated chromosomally. It should also be apparent to one skilled in the art that, for the ethanolic pathway described here, it is not necessary that the genes coding for alcohol dehydrogenase and pyruvate decarboxylase activities be under common control.

The present invention is further described below with regard to the following illustrative examples.

EXAMPLE 1. Construction of integration plasmids containing *E. coli* pyruvate formate-lyase gene

The following materials and methods were used throughout the present examples unless otherwise stated. *E. coli* TC4 was used for all genetic constructions. See Conway, T., Y.A. Osman, J.I. Konnan, E.M. Hoffman, and L.O. Ingram [1987] *J. Bacteriol.* 169: 949-954. Luria broth containing the appropriate selective antibiotic and the indicated concentrations of glucose was used in all growth experiments. Antibiotics were used in the following final concentrations except as noted: ampicillin, 50 μg/ml; chloramphenicol (Cm), 20 μg/ml or 600 μg/ml, as indicated; tetracycline, 12.5 μg/ml. ADH indicator plates containing the Schiff reagent were used to detect aldehyde produced from ethanol by recombinant *E. coli* expressing ADHII of *Z. mobilis*. Conway, T., G.W. Sewell, Y.A. Osman, and L.O. Ingram [1987] *J. Bacteriol.* 169: 2591-2597.

Standard procedures were used for plasmid preparation, restriction enzyme digestions, ligations, transformations, and gel electrophoresis. Isolation of restriction fragments for subsequent cloning was accompanied by elution from GTG agarose (FMC BioProducts, ME) using Microfilterfuge tubes (Rainin Instrument Co.).

The procedure used for construction of integration plasmids containing an *E. coli* pyruvate formate-lyase gene (*pfl*)is shown in Figure 1. Plasmid pHB4 (Sawers et al. [1988], *supra*) carrying an incomplete pyruvate formate-lyase (*pfl*) gene of *E. coli* was partially digested with BamHI, the 5'-protruding ends were filled in with the Klenow fragment of DNA polymerase I, and the fragments were rejoined by ligating to a SAlI linker (dCGTCGACG). Those in which the BamHI site outside the *pfl* gene was replaced by a SalI site were identified by screening of individual transformants. The resulting plasmid, pLOI513, contained two SalI sites which allow removal of the *pfl* portion of the plasmid from the rest of the plasmid.

A chloramphenicol resistance marker gene was excised as an 1.37-kb HhaI fragment from the plasmid cloning vector, pBR325. See Prentki, P., F. Karch, S. Iida, and J. Meyer [1981] *Gene* 14: 289-299. This HhaI fragment was treated with Klenow polymerase to fill protruding ends and ligated into the Klenow-treated BamHI site downstream from the *adhB* gene in pLOI295, see Ingram, L.O., T. Conway, D.P. Clark, G.W. Sewell, and J.F. Preton [1987] *Appl. Environ. Microbiol.* 53: 2420-2425, in the same orientation of those of *pdc* and *adhB* genes to generate pLOI515. Plasmid pLOI515 was then digested with SalI and partially digested with EcoRI, and, after a Klenow polymerization to generate blunt ends, ligated with BamHI linker (dCCGGATCCGG). The ligation mixture was separated on a GTG agarose gel. The fragment carrying promoter-less *pdc* and *adhB* genes and the Cm^r gene (with promoter) was isolated and digested with BamHI. The BamHI fragment in which EcoRI and SalI sites were regenerated on each end of the original EcoRI-SalI fragment, was cloned in to the BamHI site in the polylinker of pLOI505 generated by removing SalI site from pUC19 (available from Bethesda

Research Laboratories). The SalI site upstream from the Cm^r gene in pLOI506 was removed by digestion with SalI, filling in with Klenow polymerase and religation to generate pLOI508. The 4.6-kb BamHI fragment of pLOI508 was inserted into the BamHI site in the incomplete *pfl* structural gene carried on plasmid pLOI513. The resultant plasmid pLOI510 was used to introduce the *pdc*, *adhB*, and Cm^r genes into the chromosome of *E. coli* strain B (ATCC 11303) by homologous recombination as described below (Example 4).

EXAMPLE 2. Construction of integration plasmids containing *E. coli* pyruvate formate-lyase gene and temperature-sensitive for plasmid replication

Plasmid pLOI295 was digested with EcoRI and SalI, and the 3.2-kb EcoRI-SalI fragment carrying *pdc* and *adhB* genes was treated with Klenow fragment of DNA polymerase to produce blunt ends. This blunt-ended fragment was ligated into the Klenow-treated BamHI site of pLOI513 carrying the incomplete *pfl* gene in the same orientation with respect to transcription to give pLOI542. pLOI542 was then digested with SalI and the 7.2-kb SalI fragment was ligated to SalI site in the polylinker of pMAK705, see Hamilton, C.M., M. Aldea, B.K. Washburn, P. Babitzke, and S. R. Kushner [1989] *J. Bacteriol.* 171: 4617-4622, containing the temperature-sensitive replicon and Cm^r gene (Figure 2). The resultant plasmid pLOI543 was introduced into the *E. coli* chromosome by homologous recombination (see Example 3 below).

EXAMPLE 3. Chromosomal integration in *E. coli* B of *Z. mobilis pdc* and *adhB* genes with loss of an associated antibiotic resistance gene

The recombinant plasmid pLOI543, which replicates at 30°C, but not at 44°C, was used to

transform *E. coli* B with the Cm gene to give it resistance to chloremphenicol. Transformed cells were grown at 44°C in 100 ml Luria broth containing 20 µg/ml chloramphenicol and 5% (w/v) glucose for 24 hours to select for integration of the plasmid into the chromosome. A portion (0.1 ml) of this culture was diluted, and 0.1 ml of the diluted cell suspension was used to inoculate 100 ml of Luria broth containing 5% (w/v) glucose without chloramphenicol. The culture was grown at 30°C for 12 hours to allow excision. Two or more cycles of growth were carried out by diluting a portion (0.1 ml) of the culture and inoculating into 100 ml of fresh medium. A diluted cell suspension of the 12-hour culture was then used to inoculate Luria broth containing 5% (w/v) glucose and incubated at 44°C to eliminate plasmid. Finally, single colonies were isolated by plating serial dilutions of the culture onto Luria agar plates containing 2% (w/v) glucose and growing them at 30°C. Single colonies appearing on the plates were screened for ethanol-production genes by using aldehyde indicator plates and for loss of plasmid as sensitivity to chloramphenicol. Two clones were selected which were sensitive to chloramphenicol but contained the ethanol-production genes, strains KO1 and KO2. These clones produced pink colonies on aldehyde indicator plates.

EXAMPLE 4. Chromosomal integration in *E. coli* B of *Z. mobilis pdc* and *adhB* genes with retention of an associated antibiotic resistance gene

Plasmid pLOI510 was digested with SalI and the 8.6-kb SalI segment containing an incomplete *pfl* gene was circularized by self-ligation at a low DNA concentration. The covalently closed DNA fragment lacked sequences allowing autonomous replication. *E. coli* B was transformed with the ligation mixtures by selection for resistance to 20 µg/ml

chloramphenicol. Expression of *adhB* gene in chloramphenicol resistant transformants was confirmed by plating colonies from chloramphenicol plates to ADH indicator plates. A single clone was selected for further characterization, designated strain KO3. The lack of plasmid-borne gene for *adh* or for chloramphenicol resistance was confirmed by the lack of transformants from DNA preparations and by direct analysis on agarose gels. This clone produced pink colonies on aldehyde indicator plates.

EXAMPLE 5. Generation and detection of mutations that increase expression of *Z. mobilis pdc* and *adhB* genes in the absence of a selectable marker gene

In Example 3, chromosomal integration in *E. coli* B of *Z. mobilis pdc* and *adhB* genes was carried out under conditions that resulted in loss of the antibiotic resistance gene that was associated with those ethanol genes on the DNA segment used to transform the host cell. Strain KO2, resulting from the procedure of Example 3, was mutagenized with methyl methane sulfonate under standard conditions well known in the art of bacterial genetics. Surviving mutagenized cells (about $4 - 8 \times 10^4$) were plated on aldehyde indicator plates at approximately 200 to 400 colonies per plate. Four dark red clones indicative of increased ADHII expression were isolated and tested for ethanol production as described in Example 8 below. Of these mutants, the one with the highest yield in ethanol production was designated as strain KO20.

EXAMPLE 6. Selection for mutations that increase expression of *Z. mobilis pdc* and *adhB* genes by selection for increased expression of an associated antibiotic resistance gene

In Example 4, chromosomal integration in *E. coli* B of *Z. mobilis pdc* and *adhB* genes was carried

out under conditions that resulted in retention of the antibiotic resistance gene that was associated with those ethanol genes on the DNA segment used to transform the host cell. A series of dilutions of strain KO3, which resulted from the procedure of Example 4, were plated on Luria agar plates containing 2% glucose and 600 µg/ml chloramphenicol. Large, fat colonies were observed after overnight incubation, at a frequency of approximately 1 per 100,000 plated cells. These large colonies produced ethanol and tested bright red on alcohol indicator plates. Two strains were investigated further, strains KO4 and KO5.

EXAMPLE 7. Insertion of recA and frd mutations

The *recA* mutation in *E. coli* strain JC10240 was transferred to strains KO4 and KO5 by conjugation with selection for resistance to both Cm and tetracycline (nearby Tn10). Co-inheritance of the *recA* phenotype was confirmed by increased UV sensitivity.

An *E. coli* Hfr strain capable of mobilizing the *frd* mutation was constructed by transducing the *frd* deletion mutation from DW12 [*zid*::Tn10, Δ(*frdABCD*)] into strain KL282 with selection for Tn10. See Blaut, M., K. Whittacker, A. Valdovinos, B.A.C. Ackrell, R.P. Gunsalus and G. Cecchini [1989] *J. Biol. Chem.* 264: 13599-13604. *frd* mutants were identified among the tetracycline-resistant transductants by loss of fumarate reductase activity. The resulting Hfr strain, SE1706, was used to conjugate the *frd* deletion into strain KO4.

Tn10 was deleted from constructs by selection on a modified fusaric acid medium. See, e.g., Maloy, S.R., and W.D. Nunn [1981] *J. Bacteriology* 145: 1110-1112. This medium contained per liter: 15 g agar, 5 g tryptone, 5 g yeast extract, 20 g glucose, 10 g NaCl, 50 mg chlortetracycline, 10 g

NaH_2PO_4, 12 mg fusaric acid, and 10 mM $ZnCl_2$. Stocks of chlortetracycline (12.5 mg/ml) and fusaric acid (1 mg/ml) were prepared in 70% ethanol. Complex medium components containing chlortetracycline, sodium phosphate and zinc chloride were autoclaved separately and mixed after cooling. Antibiotics in 70% ethanol are self-sterilizing. For selection of Tn10 loss, serial dilutions of log phase cultures were spread on fusaric acid plates and incubated overnight at 37°C. Resulting colonies were streaked for isolation on additional fusaric acid plates and tested for the loss of tetracycline resistance. Three strains were constructed KO10 (*recA*), KO11 (*frd*), and KO12 (*recA*, *frd*).

EXAMPLE 8. Characterization of ethanol gene expression

Fermentation experiments: Fermentations were carried out in Luria broth supplemented with 10% (w/v) glucose or 8% (w/v) xylose. Fleakers (500-ml, Fisher Scientific Company, Orlando, FL) containing 350 ml of the medium were equipped with a pH electrode, gas outlet, and sampling port through appropriately drilled rubber caps. A Jenco model 3671 pH controller (Whatman Lab Sales, Hillsboro, OR) was used to maintain a pH of 6.0 by the addition of base (2 N KOH). Batch fermentations were carried out in duplicate at 30°C and were stirred continuously by a 1.25 inch star-shaped magnetic bar (100 rpm).

Inocula were grown overnight at 30°C from isolated colonies in unshaken flasks. Fermentations were inoculated to an initial $O.D._{550nm}$ of approximately 1.0 (330 mg dry weight of cells/liter).

Cell growth was monitored turbidimetrically at 550 nm using a Bausch and Lomb Spectronic 70 spectrophotometer. Ethanol was measured by gas-

liquid chromatography as described, for example, by Dombek, K.M., and L.O. Ingram [1986] *Appl. Environ. Microbiol.* 51: 197-200. Values for conversion efficiency were corrected for fermentation volume changes caused by the addition of base and assumed that all sugar initially added had been metabolized. Volumetric productivities and specific productivities were estimated during the early stages of fermentation (6 to 24 hours) and represent maximum values. All fermentation data in tables and in figures represent averages from two or more batch fermentations.

Analysis of volatile and non-volatile acids in the culture: Samples were removed for organic acid analyses after 72 hours of fermentation. Volatile and non-volatile acids were measured by gas-liquid chromatography with a Gow-Mac Series 580 gas chromatograph (Gow-Mac Instrument Company, Bridgewater, NJ) connected to a Hewlett-Packard 3390A integrator. Nonvolatile acids were converted to methyl esters prior to analysis.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE): Cells were grown for 24 hours under the conditions of the fermentation experiments, chilled to 0°C, harvested by centrifugation (7,000 x g, 10 minutes), washed twice with 1/3 volume of 5 mM sodium phosphate buffer (pH 6.5) containing 10 mM 2-mercaptoethanol, and stored frozen at -20°C. Cell pellets were resuspended in an equal volume of buffer and broken by two passages through a French pressure cell at 20,000 psi. Membranes were removed by centrifugation for 90 minutes at 100,000 x g. The supernatants were separated with a Biorad Mini-Protein II electrophoresis unit (Biorad Laboratories, Richmond, CA) using an 8% acrylamide denaturing sodium dodecyl sulfate gel. Protein was measured with the Bradford reagent. See Bradford, M.M. [1979] *Mol. Gen. Genet.*

181: 548-551. Approximately 20 μg of protein was loaded into each lane.

Enzyme activity: PDC activity was measured in heat-treated French press extracts as described, for example, by Conway, T., Y.A. Osman, J.I. Konnan, E.M. Hoffmann and L.O. Ingram [1987] *J. Bacteriol.* 169: 949-954. Heat treatment was used to inactivate competing native enzymes which complicate measurements of PDC in recombinant *E. coli*.

It should be understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and the scope of the appended claims.

What is claimed is:

1. A recombinant host cell comprising a chromosome comprised of

(a) a heterologous DNA segment under transcriptional control of a promoter which is endogenous to said host cell, wherein said DNA segment encodes a desired polypeptide; and

(b) a mutation that causes increased expression of said heterologous DNA segment, resulting in increased production by said host cell of said polypeptide compared to production of said polypeptide by said host cell in the absence of said mutation, wherein said increased expression is retained in the absence of conditions that select for cells having said increased expression.

2. A recombinant host cell according to claim 1, wherein said cell is a microbial cell.

3. A recombinant host cell according to claim 1, wherein said heterologous DNA segment encodes a plurality of genes.

4. A recombinant host cell according to claim 3, wherein said plurality of genes comprises a selectable marker gene.

5. A recombinant host cell according to claim 4, wherein expression of said selectable marker gene confers antibiotic resistance upon said cell.

6. A recombinant host cell according to claim 5, wherein expression of said marker gene confers chloramphenicol resistance upon said cell.

7. A recombinant host cell according to claim 2, wherein said cell is an enteric bacterium.

8. A recombinant host cell according to claim 7, wherein said enteric bacterium is selected from a strain of *Erwinia chrysanthemi*, *Escherichia coli*, or *Klebsiella pneumoniae*

9. A recombinant host cell according to claim 7, wherein said enteric bacterium is a strain of *Escherichia coli*.

10. A recombinant host cell according to claim 1, wherein said heterologous DNA segment is under transcriptional control of a strong endogenous promoter.

11. A recombinant host cell according to claim 10, wherein said promoter is a pyruvate formate-lyase (*pfl*) promoter.

12. A recombinant host cell according to claim 1, wherein said heterologous DNA segment encodes an alcohol dehydrogenase and a pyruvate decarboxylase.

13. A recombinant host cell according to claim 12, wherein said alcohol dehydrogenase and said pyruvate decarboxylase are encoded by genes from *Zymomonas mobilis*.

14. A recombinant host cell according to claim 13, wherein said cell is able to produce ethanol by fermentation of glucose or xylose with a theoretical yield corresponding to conversion of at least about 100% of added sugar to ethanol.

15. A recombinant host cell according to claim 12, wherein said chromosome further comprises a mutation that impairs succinate production.

16. A recombinant host cell according to claim 15, wherein said mutation that impairs succinate production comprises a mutation in a fumarate reductase (*frd*) gene.

17. A recombinant host cell according to claim 1, wherein said chromosome further comprises a mutation that impairs recombination in said host cell.

18. A recombinant host cell according to claim 17, wherein said mutation that impairs recombination comprises a mutation in a *recA* gene.

19. A cell strain that is the product of a process comprising the steps of:

(a) providing a culture comprised of host cells comprising a chromosome that encodes a promoter endogenous to said host cells and a host gene under transcriptional control of said promoter;

(b) transforming host cells in said culture with a heterologous DNA segment comprising

(i) a plurality of genes including a selectable marker gene and a gene encoding a desired polypeptide, and

(ii) sequences that are sufficiently homologous to said host gene and properly located in said heterologous DNA segment to enable integration into said host gene of said plurality of genes encoded by said heterologous DNA segment by means of

homologous recombination;

(c) selecting for host cells in said culture, or progeny thereof, that express said selectable marker gene at a first level;

(d) screening host cells selected in step (c), or progeny thereof, to obtain host cells that produce said desired polypeptide at an initial level;

(e) optionally exposing host cells identified in step (d), or progeny thereof, to a mutagen under conditions such that mutations are created in said chromosome; and then

(f) testing host cells produced in step (d) or step (e), or progeny thereof, for host cells that produce said desired protein at a level higher than said initial level, to obtain host cells having a mutation that causes increased expression of said heterologous DNA segment resulting in an increase in production by said host cells of said desired polypeptide compared to said production of said desired polypeptide by said host cells in the absence of said mutation, wherein said increased expression is retained in the absence of conditions that select for cells having said increased expression.

20. A cell strain according to claim 19, wherein further

(i) in step (b) said heterologous DNA segment further comprises a plasmid, wherein said plasmid comprises a replicon that is temperature-sensitive for replication;

(ii) in step (b) said transforming host cells further comprises introducing said heterologous DNA segment into said host cells

and growing said host cells under conditions that select for cells that express said selectable marker gene at said first level and at a temperature that does not permit replication of said plasmid, resulting in integration of said heterologous DNA segment into said host gene of said chromosome by homologous recombination; and

(iii) in step (c) said selecting for host cells further comprises growing said host cells that express said selectable marker gene at said first level under conditions that do not select for cells that express said selectable marker gene, wherein said host cells are grown under said conditions at a first temperature that does permit replication of said plasmid, resulting in excision from said host gene of said selectable marker gene and of said plasmid, and wherein further then said host cells are grown under said conditions at a second temperature that does not permit replication of said plasmid, resulting in host cells that retain said gene encoding said desired polypeptide in the absence of said selectable marker gene and said plasmid.

21. A cell strain according to claim 19, wherein further

(i) in step (b) said heterologous DNA segment comprises a closed circular DNA lacking an ability to replicate, and

(ii) in step (f) said testing host cells comprises selecting for host cells produced in step (d) or step (e), or progeny thereof, that express said selectable marker gene at a second that is level higher than said first level, and then screening said host cells that express said selectable marker gene at said second level for host cells that produce said desired protein at a level higher than said initial level.

22. A cell strain according to claim 19, wherein said host cell strain is a microbial cell strain.

23. A strain according to claim 19, wherein said selectable marker protein confers resistance to chloramphenicol on said host cell strain.

24. A cell strain according to claim 19, wherein said first level of expression of said selectable marker gene confers resistance to at least about 20 μg/ml of chloramphenicol

25. A cell strain according to claim 22, wherein said first level of expression of said selectable marker gene confers resistance to at least about 20 μg/ml of chloramphenicol and said second level of expression of said selectable marker protein confers resistance to at least about 100 μg/ml of chloramphenicol.

26. A cell strain according to claim 19,

wherein said selectable marker gene is under the transcriptional control of a promoter located in said heterologous DNA segment downstream of said gene encoding said desired polypeptide.

27. A cell strain according to claim 26, wherein said heterologous DNA segment further comprises a transcription terminator, said transcription terminator being located between said gene encoding said desired polypeptide and said selectable marker gene.

28. A cell strain according to claim 19, wherein said host cell strain is an enteric bacterial strain.

29. A cell strain according to claim 19, wherein said enteric bacterial strain is a strain of *Escherichia coli*.

30. A cell strain according to claim 19, wherein said promoter is a strong promoter.

31. A cell strain according to claim 30, wherein said promoter is a pyruvate formate-lyase (*pfl*) promoter.

32. A cell strain according to claim 19, wherein said heterologous DNA segment encodes an alcohol dehydrogenase and an pyruvate decarboxylase.

33. A cell strain according to claim 32, wherein said alcohol dehydrogenase and said pyruvate decarboxylase are encoded by genes from *Zymomonas mobilis*.

34. A cell strain according to claim 33, wherein said strain is able to produce ethanol by

fermentation of glucose or xylose with a theoretical yield corresponding to conversion of at least about 100% of added sugar to ethanol.

35. A cell strain according to claim 32, wherein said chromosome further comprises a mutation that impairs succinate production.

36. A cell strain according to claim 35, wherein said mutation that impairs succinate production comprises a mutation in a fumarate reductase (*frd*) gene.

37. A cell strain according to claim 19, wherein said chromosome further comprises a mutation that impairs recombination in said host cell strain.

38. A cell strain according to claim 37, wherein said mutation that impairs recombination comprises a mutation in a *recA* gene.

39. A process for producing a recombinant host cell strain that produces high levels of a desired polypeptide, comprising the steps of:

(a) providing a culture comprised of host cells comprising a chromosome that encodes a promoter endogenous to said host cells and a host gene under transcriptional control of said promoter;

(b) transforming host cells in said culture with a heterologous DNA segment comprising

(i) a plurality of genes including a selectable marker gene and a gene encoding said desired polypeptide, and

(ii) sequences that are sufficiently homologous to said host gene and properly located in said

heterologous DNA segment to enable integration into said host gene of said plurality of genes encoded by said heterologous DNA segment by means of homologous recombination;

(c) selecting for host cells in said culture, or progeny thereof, that express said selectable marker gene at a first level;

(d) screening host cells selected in step (c), or progeny thereof, to obtain host cells that produce said desired polypeptide at an initial level;

(e) optionally exposing host cells identified in step (d), or progeny thereof, to a mutagen under conditions such that mutations are created in said chromosome; and then

(f) testing host cells produced in step (d) or step (e), or progeny thereof, for host cells that produce said desired protein at a level higher than said initial level, to obtain host cells having a mutation that causes increased expression of said heterologous DNA segment resulting in an increase in production by said host cells of said desired polypeptide compared to said production of said desired polypeptide by said host cells in the absence of said mutation, wherein said increased expression is retained in the absence of conditions that select for cells having said increased expression.

40. The process according to claim 39, wherein further

(i) in step (b) said heterologous DNA segment further comprises a plasmid, wherein said plasmid comprises a replicon that is

(iii) in step (c) said selecting for host cells further comprises growing said host cells that express said selectable marker gene at said first level under conditions that do not select for cells that express said selectable marker gene, wherein said host cells are grown under said conditions at a first temperature that does permit replication of said plasmid, resulting in excision from said host gene of said selectable marker gene and of said plasmid, and wherein further then said host cells are grown under said conditions at a second temperature that does not permit replication of said plasmid, resulting in host cells that retain said gene encoding said desired polypeptide in the absence of said selectable marker gene and said plasmid.

41. A process according to claim 39, wherein further

(i) in step (b) said heterologous DNA segment comprises a closed circular DNA lacking an ability to replicate, and

(ii) in step (f) said testing host cells comprises selecting for host cells produced in step (d) or step (e), or progeny thereof, that express said selectable marker gene at a second that is level higher than said first level, and then screening said host cells that express said selectable marker gene at said second level for host cells that produce said desired protein at a level higher than said initial level.

42. A recombinant host cell strain according to claim 39, wherein said host cell strain is a microbial host cell strain.

43. A process according to claim 39, wherein said selectable marker protein confers resistance to chloramphenicol on said host cell strain.

44. A process according to claim 43, wherein said first level of expression of said selectable marker gene confers resistance to at least about 20 μg/ml of chloramphenicol.

45. A process according to claim 43, wherein said first level of expression of said selectable marker gene confers resistance to at least about 20 μg/ml of chloramphenicol and said second level of expression of said selectable marker protein confers resistance to at least about 100 μg/ml of chloramphenicol.

46. A process according to claim 39, wherein said selectable marker gene is under the transcriptional control of a promoter located in said heterologous DNA segment downstream of said gene encoding said desired polypeptide.

47. A process according to claim 46, wherein said heterologous DNA segment further comprises a transcription terminator, said transcription terminator being located between said gene encoding said desired polypeptide and said selectable marker gene.

48. A process according to claim 39, wherein said host cell strain is an enteric bacterial strain.

49. A process according to claim 39, wherein said enteric host cell strain is a strain of *Escherichia coli*.

50. A process according to claim 39, wherein said promoter is a strong promoter.

51. A process according to claim 50, wherein said promoter is a pyruvate formate-lyase (*pfl*) promoter.

52. A process according to claim 39, wherein said heterologous DNA segment encodes an alcohol dehydrogenase and an pyruvate decarboxylase.

53. A process according to claim 51, wherein said alcohol dehydrogenase and said pyruvate decarboxylase are encoded by genes from *Zymomonas mobilis*.

54. A process according to claim 53, wherein said strain is able to produce ethanol by fermentation of glucose or xylose with a theoretical yield corresponding to conversion of at least about 100% of added sugar to ethanol.

55. A process according to claim 51, wherein said chromosome further comprises a mutation that impairs succinate production.

56. A process according to claim 55, wherein said mutation that impairs succinate production comprises a mutation in a fumarate reductase (*frd*) gene.

57. A process according to claim 39, wherein said chromosome further comprises a mutation that impairs recombination in said host cell strain.

58. A process according to claim 57, wherein said mutation that impairs recombination comprises a mutation in a *recA* gene.

59. The recombinant host strain of *Escherichia coli* (pLOI510) represented by a deposit with the American Type Culture Collection designated as deposit number ATCC 68484.

60. The recombinant host strain of Escherichia coli (pLOI543) represented by a deposit with the American Type Culture Collection designated as deposit number ATCC 68485.

61. The recombinant host strain, according to claim 19, of *Escherichia coli* KO4 represented by a deposit with the American Type Culture Collection designated as deposit number ATCC 55123.

62. The recombinant host strain, according to claim 19, of *Escherichia coli* KO11 represented by a deposit with the American Type Culture Collection designated as deposit number ATCC 55124.

63. The recombinant host strain, according to claim 19, of *Escherichia coli* KO12 represented by a deposit with the American Type Culture Collection designated as deposit number ATCC 55125.

64. The recombinant host strain, according to claim 19, of *Escherichia coli* KO20 represented by a deposit with the American Type Culture Collection designated as deposit number ATCC 55126.

AMENDED CLAIMS

[received by the International Bureau on 23 May 1992 (23.05.92);
original claims 2,7,22 and 42 amended; new claims 61-66 added;
remaining claims renumbered as claims 1-60 (13 pages)]

1. A recombinant host cell comprising a chromosome comprised of

(a) a heterologous DNA segment that is integrated into a host gene which is under transcriptional control of a strong promoter, wherein said host gene and said promoter are endogenous to said host cell and wherein said DNA segment encodes a desired polypeptide; and

(b) a mutation that causes increased expression of said heterologous DNA segment, resulting in increased production by said host cell of said polypeptide compared to production of said polypeptide by said host cell in the absence of said mutation, wherein said increased expression is retained in the absence of conditions that select for cells having said increased expression.

2. A recombinant host cell according to claim 1, wherein said heterologous DNA segment comprises a plurality of genes.

3. A recombinant host cell according to claim 2, wherein said plurality of genes comprises a selectable marker gene.

4. A recombinant host cell according to claim 3, wherein expression of said selectable marker gene confers antibiotic resistance upon said cell.

5. A recombinant host cell according to claim 4, wherein expression of said marker gene confers chloramphenicol resistance upon said cell.

6. A recombinant host cell according to claim 1, wherein said cell is an enteric bacterial host cell.

7. A recombinant host cell according to claim 6, wherein said enteric bacterial host cell is selected from the group consisting of *Erwinia chrysanthemi*, *Escherichia coli* and *Klebsiella pneumoniae*.

8. A recombinant host cell according to claim 7, wherein said enteric bacterial host cell is an *Escherichia coli* cell.

9. A recombinant host cell according to claim 1, wherein said heterologous DNA segment is under transcriptional control of an endogenous promoter of a gene that is expressed at substantially the level at which the pyruvate formate-lyase (*pfl*) gene is expressed in said host cell.

10. A recombinant host cell according to claim 9, wherein said promoter is a promoter of a pyruvate formate-lyase (*pfl*) gene.

11. A recombinant host cell according to claim 1, wherein said heterologous DNA segment encodes an alcohol dehydrogenase and a pyruvate decarboxylase.

12. A recombinant host cell according to claim 11, wherein said alcohol dehydrogenase and said pyruvate decarboxylase are encoded by genes from *Zymomonas mobilis*.

13. A recombinant host cell according to claim 12, wherein said cell is an *Escherichia coli* cell, said promoter is a promoter of the pyruvate formate-lyase (*pfl*) gene, and said cell is able to produce ethanol by fermentation of glucose or xylose with a theoretical yield corresponding to conversion of at least about 100% of added sugar to ethanol.

14. A recombinant host cell according to claim 13, wherein said chromosome further comprises a mutation that impairs succinate production.

15. A recombinant host cell according to claim 14, wherein said mutation that impairs succinate production comprises a mutation in a fumarate reductase (*frd*) gene.

16. A recombinant host cell according to claim 8, wherein said chromosome further comprises a mutation that impairs recombination in said host cell.

17. A recombinant host cell according to claim 16, wherein said mutation that impairs recombination comprises a mutation in a *recA* gene.

18. A recombinant host cell strain that is the product of a process comprising the steps of:

(a) providing a culture comprised of host cells comprising a chromosome that encodes a strong promoter endogenous to said host cells and a host gene under transcriptional control of said promoter;

(b) transforming host cells in said culture with a DNA molecule comprising

(i) a heterologous DNA segment comprising a plurality of genes including a selectable marker gene and a gene encoding a desired polypeptide, and

(ii) sequences that flank said heterologous DNA segment and are homologous to said host gene,

whereby integration into said host gene of said heterologous DNA segment results by means of homologous recombination;

(c) selecting for host cells produced in step (b) that express said selectable marker gene at a first level;

(d) screening host cells obtained in step (c) to obtain host cells that produce said desired polypeptide at an initial level;

(e) optionally exposing host cells identified in step (d) to a mutagen under conditions such that mutations are created in said chromosome; and then

(f) testing host cells produced in step (d) or step (e) for host cells that produce said desired protein at a level higher than said initial level, to obtain host cells having a mutation that causes increased expression of said heterologous DNA segment resulting in an increase in production by said host cells of said desired polypeptide compared to said production of said desired polypeptide by said host cells in the absence of said mutation, wherein said increased expression is retained in the absence of conditions that select for cells having said increased expression.

19. A strain according to claim 18, wherein said strain is a strain of *Escherichia coli* and wherein further

(i) in step (b) said DNA molecule is a plasmid, wherein said plasmid comprises a replicon that is temperature-sensitive for replication;

(ii) in step (b) said transforming host cells further comprises introducing said plasmid into said host cells and growing said host cells under conditions that select for cells that express said selectable marker gene at said first level and at a temperature that does not permit replication of said plasmid, resulting in integration of said plasmid into said host gene of said chromosome by homologous recombination; and

(iii) in step (c) said selecting for host cells further comprises

(1) growing said host cells that express said selectable marker gene at said first level

under conditions that do not select for cells that express said selectable marker gene, wherein said host cells are grown under said conditions at a first temperature that does permit replication of said plasmid, resulting in excision from said host gene of said selectable marker gene and of said plasmid, and wherein further

(2) said host cells are grown under said conditions at a second temperature that does not permit replication of said plasmid, resulting in host cells that retain said gene encoding said desired polypeptide in the absence of said selectable marker gene and said plasmid.

20. A strain according to claim 18, wherein further

(i) in step (b) said DNA molecule comprises a closed circular DNA lacking an ability to replicate, and

(ii) in step (f) said testing host cells comprises selecting for host cells produced in step (d) or step (e) that express said selectable marker gene at a second that is higher than said first level, and then screening said host cells that express said selectable marker gene at said second level for host cells that produce said desired protein at a level higher than said initial level.

21. A strain according to claim 18, wherein said selectable marker protein confers resistance to chloramphenicol on said host strain.

22. A strain according to claim 21, wherein said first level of expression of said selectable marker gene confers resistance to at least about 20 μg/ml of chloramphenicol

23. A strain according to claim 21, wherein said first level of expression of said selectable marker gene confers resistance to at least about 20 μg/ml of chloramphenicol and said second level of expression of said selectable marker protein confers resistance to at least about 100 μg/ml of chloramphenicol.

24. A strain according to claim 18, wherein said selectable marker gene is under the transcriptional control of a promoter located in said heterologous DNA segment downstream of said gene encoding said desired polypeptide.

25. A strain according to claim 24, wherein said heterologous DNA segment further comprises a transcription terminator, said transcription terminator being located between said gene encoding said desired polypeptide and said selectable marker gene.

26. A strain according to claim 18, wherein said host cells are enteric bacterial host cells.

27. A strain according to claim 26, wherein said enteric bacterial host cells are *Erwinia chrysanthemi*, *Escherichia coli* or *Klebsiella pneumoniae* cells.

28. A strain according to claim 27, wherein said enteric bacterial host cells are *Escherichia coli* cells.

29. A strain according to claim 18, wherein said promoter is an endogenous promoter of a gene that is expressed at substantially the level at which the pyruvate formate-lyase (*pfl*) gene is expressed in said host cell.

30. A strain according to claim 29 wherein said promoter is a promoter of a pyruvate formate-lyase (*pfl*) gene.

31. A strain according to claim 18, wherein said heterologous DNA segment encodes an alcohol dehydrogenase and a pyruvate decarboxylase.

32. A strain according to claim 31, wherein said alcohol dehydrogenase and said pyruvate decarboxylase are encoded by genes from *Zymomonas mobilis*.

33. A strain according to claim 32, wherein said strain is an *Escherichia coli* strain, said promoter is a promoter of the pyruvate formate-lyase (*pfl*) gene, and said strain is able to produce ethanol by fermentation of glucose or xylose with a theoretical yield corresponding to conversion of at least about 100% of added sugar to ethanol.

34. A strain according to claim 28, wherein said chromosome further comprises a mutation that impairs succinate production.

35. A strain according to claim 34, wherein said mutation that impairs succinate production comprises a mutation in a fumarate reductase (*frd*) gene.

36. A strain according to claim 28, wherein said chromosome further comprises a mutation that impairs recombination in said host strain.

37. A strain according to claim 36, wherein said mutation that impairs recombination comprises a mutation in a *recA* gene.

38. A process for producing a recombinant host cell strain that produces high levels of a desired polypeptide, comprising the steps of:

(a) providing a culture comprised of host cells comprising a chromosome that encodes a strong

promoter endogenous to said host cells and a host gene under transcriptional control of said promoter;

(b) transforming host cells in said culture with a DNA molecule comprising

(i) a heterologous DNA segment comprising a plurality of genes including a selectable marker gene and a gene encoding a desired polypeptide, and

(ii) sequences that flank said heterologous DNA segment and are homologous to said host gene;

whereby integration into said host gene of said heterologous DNA segment results by means of homologous recombination;

(c) selecting for host cells produced in step (b) that express said selectable marker gene at a first level;

(d) screening host cells obtained in step (c) to obtain host cells that produce said desired polypeptide at an initial level;

(e) optionally exposing host cells identified in step (d) to a mutagen under conditions such that mutations are created in said chromosome; and then

(f) testing host cells produced in step (d) or step (e) for host cells that produce said desired protein at a level higher than said initial level, to obtain host cells having a mutation that causes increased expression of said heterologous DNA segment resulting in an increase in production by said host cells of said desired polypeptide compared to said production of said desired polypeptide by said host cells in the absence of said mutation, wherein said increased expression is retained in the absence of conditions that select for cells having said increased expression.

39. The process according to claim 38, wherein said strain is a strain of *Escherichia coli* and wherein further

(i) in step (b) said DNA molecule is a plasmid, wherein said plasmid comprises a replicon that is temperature-sensitive for replication;

(ii) in step (b) said transforming host cells further comprises introducing said plasmid into said host cells and growing said host cells under conditions that select for cells that express said selectable marker gene at said first level and at a temperature that does not permit replication of said plasmid, resulting in integration of said plasmid into said host gene of said chromosome by homologous recombination; and

(iii) in step (c) said selecting for host cells further comprises

(1) growing said host cells that express said selectable marker gene at said first level under conditions that do not select for cells that express said selectable marker gene, wherein said host cells are grown under said conditions at a first temperature that does permit replication of said plasmid, resulting in excision from said host gene of said selectable marker gene and of said plasmid, and wherein further

(2) said host cells are grown under said conditions at a second temperature that does not permit replication of said plasmid, resulting in host cells that retain said gene encoding said desired polypeptide in the absence of said selectable marker gene and said plasmid.

40. A process according to claim 38, wherein further (i) in step (b) said DNA molecule comprises a closed circular DNA lacking an ability to replicate, and (ii) in step (f) said testing host cells comprises selecting for host cells produced in step (d) or step (e) that express said selectable marker gene at a second that is higher than said first level, and then screening said host cells that express said selectable marker gene at said second level for host cells that produce said desired protein at a level higher than said initial level.

41. A process according to claim 38, wherein said selectable marker protein confers resistance to chloramphenicol on said host strain.

42. A process according to claim 41, wherein said first level of expression of said selectable marker gene confers resistance to at least about 20 μg/ml of chloramphenicol.

43. A process according to claim 41, wherein said first level of expression of said selectable marker gene confers resistance to at least about 20 μg/ml of chloramphenicol and said second level of expression of said selectable marker protein confers resistance to at least about 100 μg/ml of chloramphenicol.

44. A process according to claim 38, wherein said selectable marker gene is under the transcriptional control of a promoter located in said heterologous DNA segment downstream of said gene encoding said desired polypeptide.

45. A process according to claim 44, wherein said heterologous DNA segment further comprises a transcription terminator, said transcription terminator

being located between said gene encoding said desired polypeptide and said selectable marker gene.

46. A process according to claim 38, wherein said host cells are enteric bacterial cells.

47. A process according to claim 46, wherein said enteric bacterial cells are *Erwinia chrysanthemi*, *Escherichia coli* or *Klebsiella pneumoniae* cells.

48. A process according to claim 46, wherein said host cells are *Escherichia coli* cells.

49. A process according to claim 38 wherein said promoter is an endogenous promoter of a gene that is expressed at substantially the level at which the pyruvate formate-lyase (*pfl*) gene is expressed in said host cell.

50. A process according to claim 49, wherein said promoter is a pyruvate formate-lyase (*pfl*) promoter.

51. A process according to claim 38, wherein said heterologous DNA segment encodes an alcohol dehydrogenase and an pyruvate decarboxylase.

52. A process according to claim 51, wherein said alcohol dehydrogenase and said pyruvate decarboxylase are encoded by genes from *Zymomonas mobilis*.

53. A process according to claim 52, wherein said strain is an *Escherichia coli* strain, said promoter is a promoter of the pyruvate formate-lyase (*pfl*) gene, and said strain is able to produce ethanol by fermentation of glucose or xylose with a theoretical yield corresponding to conversion of at least about 100% of added sugar to ethanol.

54. A process according to claim 48, wherein said chromosome further comprises a mutation that impairs succinate production.

55. A process according to claim 54, wherein said mutation that impairs succinate production comprises a mutation in a fumarate reductase (*frd*) gene.

56. A process according to claim 48, wherein said chromosome further comprises a mutation that impairs recombination in said host strain.

57. A process according to claim 56, wherein said mutation that impairs recombination comprises a mutation in a *recA* gene.

58. The recombinant host strain of *Escherichia coli* (pLOI510) represented by a deposit with the American Type Culture Collection designated as deposit number ATCC 68484.

59. The recombinant host strain of Escherichia coli (pLOI543) represented by a deposit with the American Type Culture Collection designated as deposit number ATCC 68485.

60. The recombinant host strain, according to claim 18, of *Escherichia coli* KO4 represented by a deposit with the American Type Culture Collection designated as deposit number ATCC 55123.

61. The recombinant host strain, according to claim 18, of *Escherichia coli* KO11 represented by a deposit with the American Type Culture Collection designated as deposit number ATCC 55124.

62. The recombinant host strain, according to claim 18, of *Escherichia coli* KO12 represented by a deposit

with the American Type Culture Collection designated as deposit number ATCC 55125.

63. The recombinant host strain, according to claim 18, of *Escherichia coli* KO20 represented by a deposit with the American Type Culture Collection designated as deposit number ATCC 55126.

64. A recombinant host cell according to claim 12, wherein said promoter is a promoter of a pyruvate formate-lyase (*pfl*) gene and said cell is able to produce ethanol by fermentation of glucose or xylose with a theoretical yield corresponding to conversion of at least about 90% of added sugar to ethanol.

65. A strain according to claim 32, wherein said promoter is a promoter of a pyruvate formate-lyase (*pfl*) gene and said cell is able to produce ethanol by fermentation of glucose or xylose with a theoretical yield corresponding to conversion of at least about 90% of added sugar to ethanol.

66. A process according to claim 51, wherein said promoter is a promoter of a pyruvate formate-lyase (*pfl*) gene and said cell is able to produce ethanol by fermentation of glucose or xylose with a theoretical yield corresponding to conversion of at least about 90% of added sugar to ethanol.

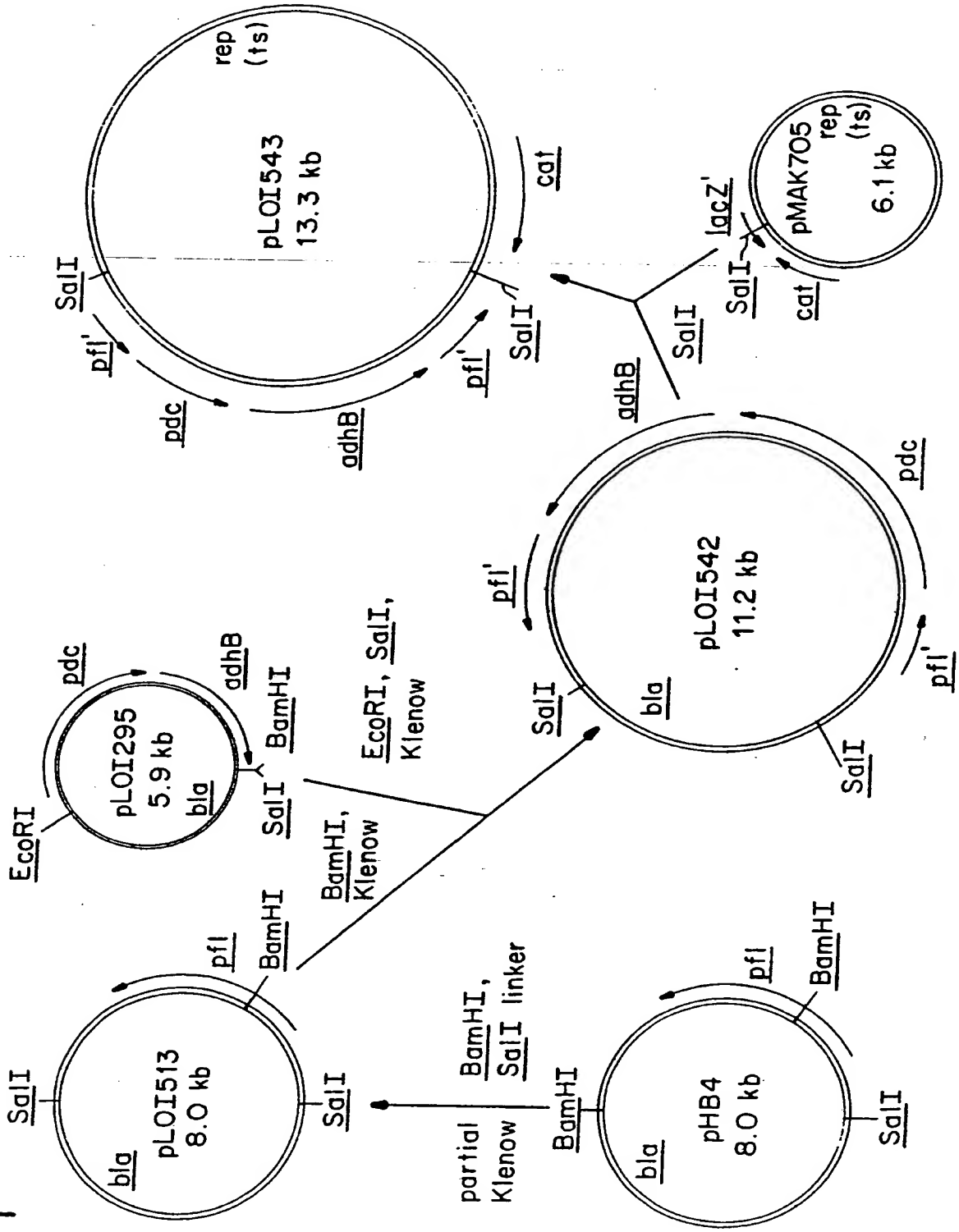
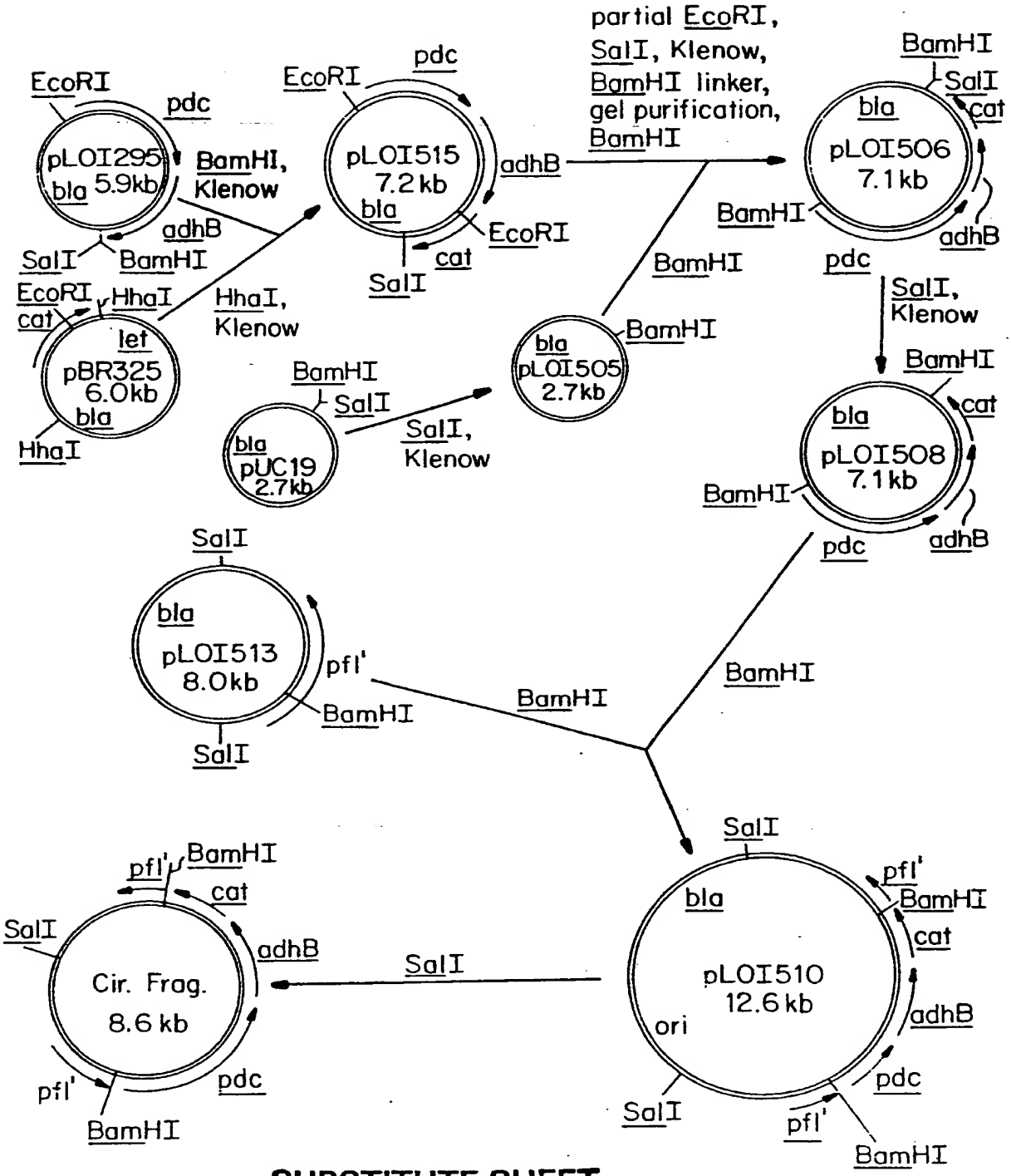


FIG. 1

SUBSTITUTE SHEET

FIG. 2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pdc
pLOI295
bla 5.9kb
BamHI, Klenow
adhB
SalI
BamHI
EcoRI
HhaI
cat
let
pBR325
6.0kb
bla
HhaI
HhaI, Klenow
EcoRI
pdc
pLOI515
7.2kb
bla
adhB
EcoRI
cat
SalI
partial EcoRI, SalI, Klenow, BamHI linker, gel purification, BamHI
BamHI
SalI
cat
pLOI506
7.1kb
BamHI
adhB
pdc
SalI, Klenow
BamHI
bla
pLOI505
2.7kb
BamHI
BamHI
SalI
bla
pUC19
2.7kb
SalI, Klenow
BamHI
cat
bla
pLOI508
7.1kb
BamHI
adhB
pdc
SalI
bla
pLOI513
8.0kb
pfl'
BamHI
SalI
BamHI
BamHI
SalI
bla
pLOI510
12.6kb
ori
pfl'
BamHI
cat
adhB
pdc
SalI
pfl'
BamHI
pfl'
BamHI
cat
SalI
adhB
Cir. Frag.
8.6 kb
SalI
pfl'
pdc
BamHI

FIG. 3A

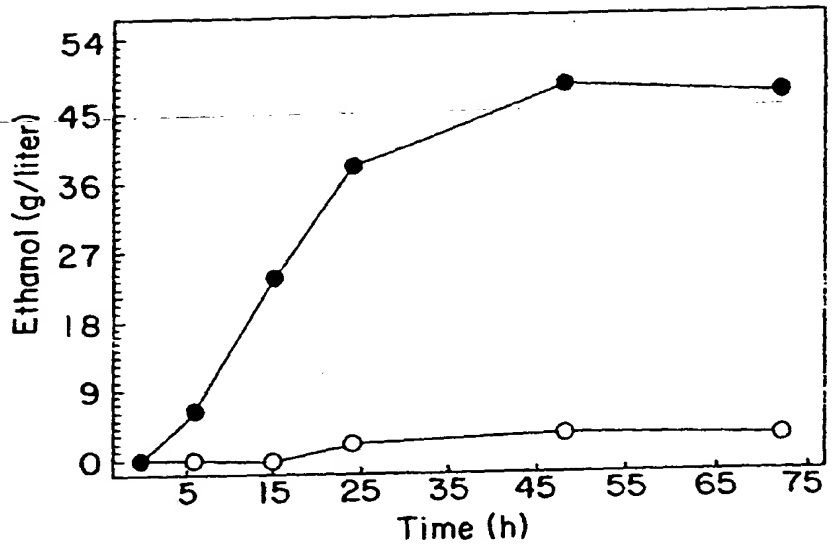

Ethanol (g/liter)
54
45
36
27
18
9
0
5
15
25
35
45
55
65
75
Time (h)

FIG. 3B

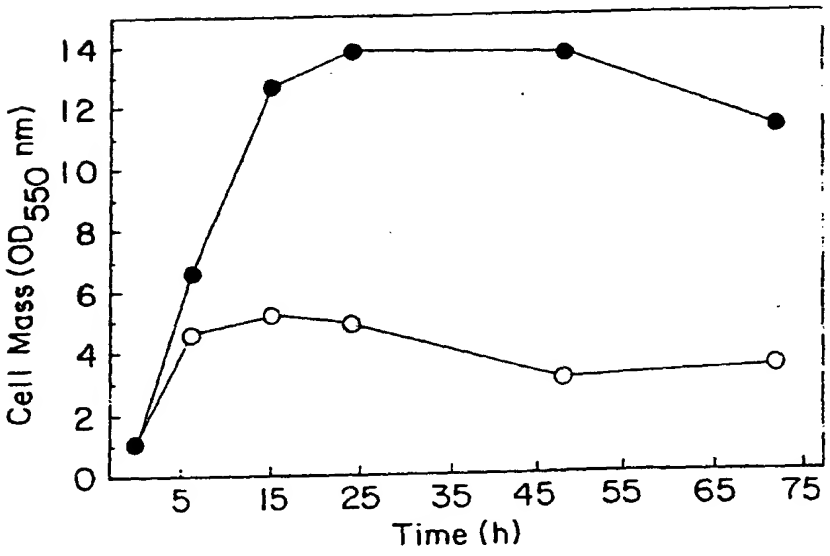

Cell Mass (OD550 nm)
14
12
10
8
6
4
2
0
5
15
25
35
45
55
65
75
Time (h)

FIG. 3C

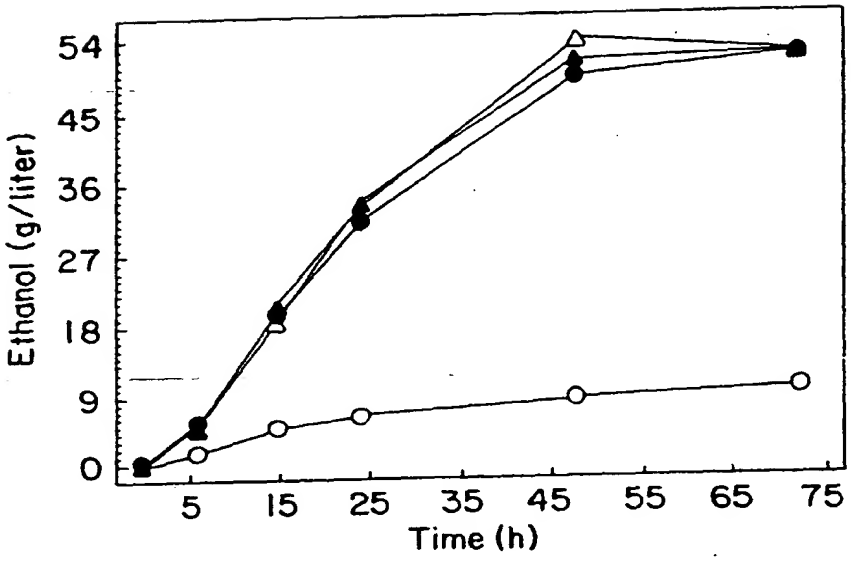

Ethanol (g/liter)
54
45
36
27
18
9
0
5
15
25
35
45
55
65
75
Time (h)

FIG. 3D

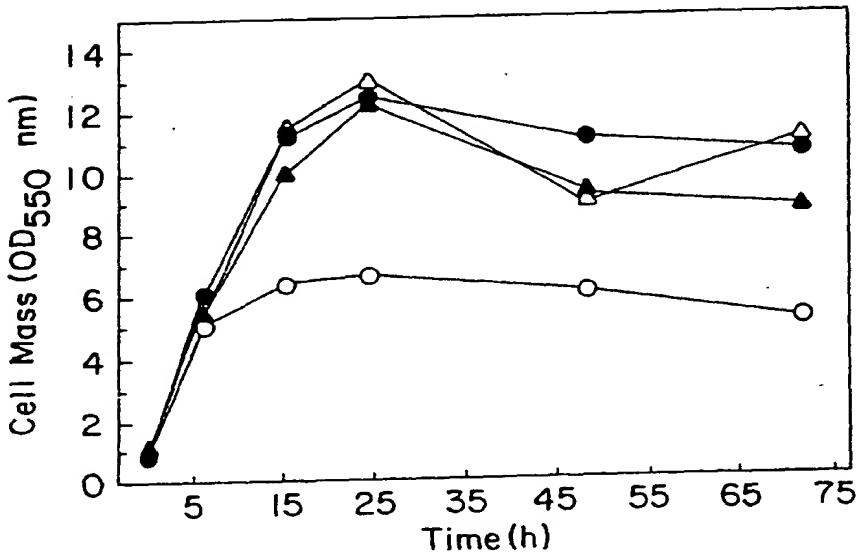

Cell Mass (OD550 nm)
14
12
10
8
6
4
2
0
5
15
25
35
45
55
65
75
Time (h)

FIG. 3E

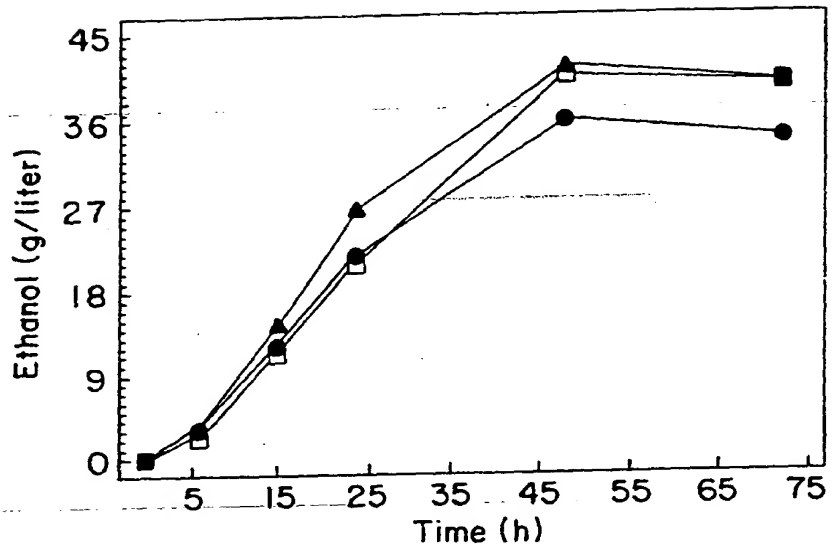

Ethanol (g/liter)
0
9
18
27
36
45
5
15
25
35
45
55
65
75
Time (h)

FIG. 3F

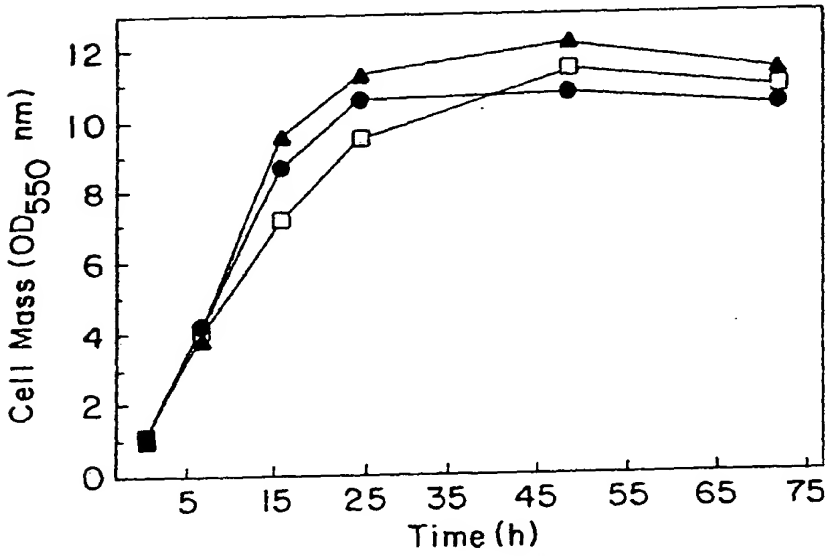

Cell Mass (OD_{550} nm)
0
2
4
6
8
10
12
5
15
25
35
45
55
65
75
Time (h)

FIG. 3G

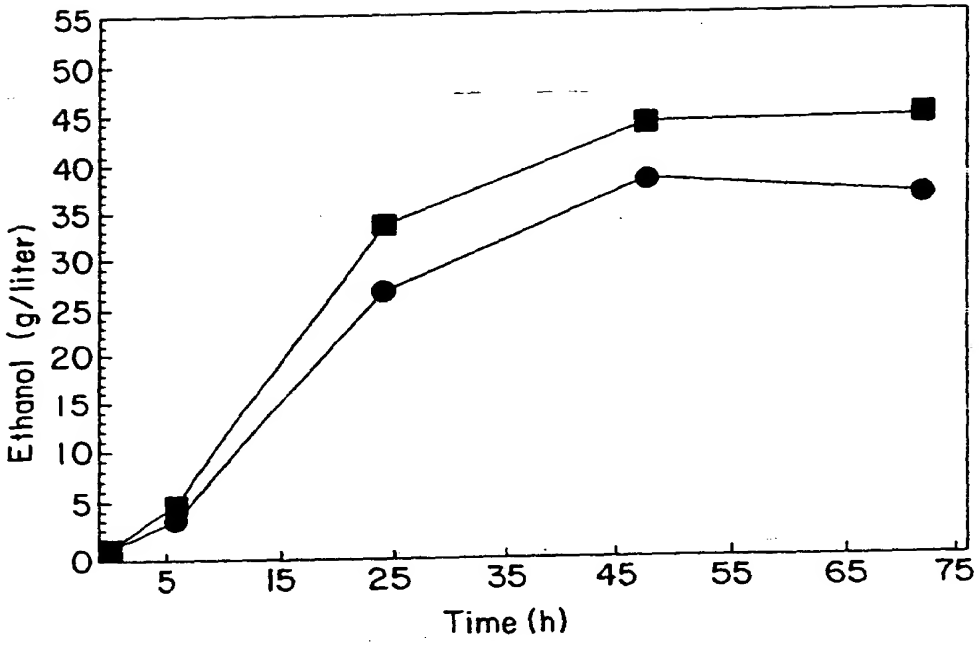

Ethanol (g/liter)
55
50
45
40
35
30
25
20
15
10
5
0
5
15
25
35
45
55
65
75
Time (h)

FIG. 4A

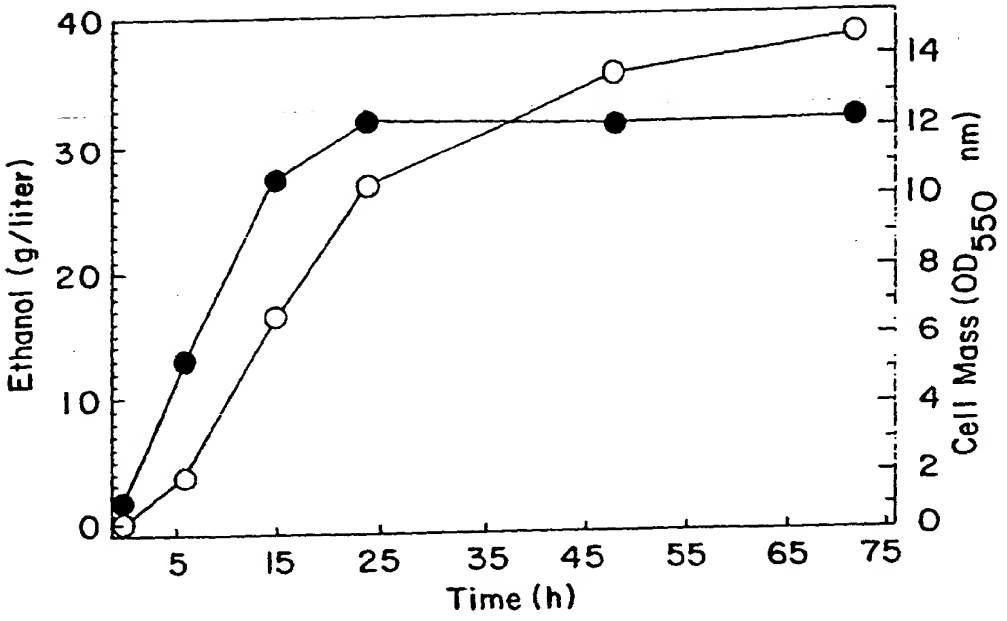
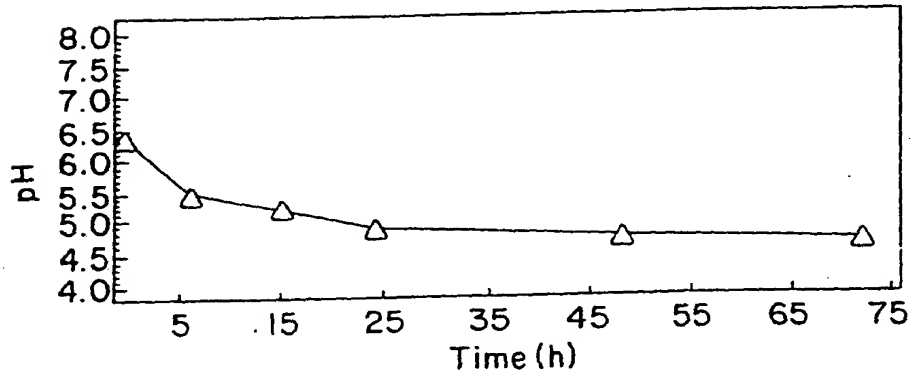

Ethanol (g/liter)
Cell Mass (OD_{550} nm)
Time (h)
0
10
20
30
40
0
2
4
6
8
10
12
14
5
15
25
35
45
55
65
75

FIG. 4B


pH
Time (h)
4.0
4.5
5.0
5.5
6.0
6.5
7.0
7.5
8.0
5
15
25
35
45
55
65
75

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/08835

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC (5): C12N 1/00,1/21,1/19,15/01,15/10,15/31,15/05,15/04,15/05,15/06
US CL : 435/252.3, 252.33, 240.2, 161, 172.3

II. FIELDS SEARCHED

Minimum Documentation Searched[4]

Classification System	Classification Symbols
U.S.	435/252.3, 252.33, 240.2, 161, 172.3

Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched[5]

Databases: Dialog (Files 5, 350,351) and APS

III. DOCUMENTS CONSIDERED TO BE RELEVANT[14]

Category*	Citation of Document,[16] with indication, where appropriate, of the relevant passages[17]	Relevant to Claim No.[18]
Y	BIOTECHNOLOGY & BIOENGINEERING, Volume 33, issued March 1989, G.K. Whitney et al., "Induction of T4 DNA ligase in a Recombinant Strain of Escherichia coli", pages 991-998, see entire document.	1-64
Y	R.W. OLD et al., "PRINCIPLES OF GENE MANIPULATIONS", published 1985 by BLACKWELL PUBLICATIONS (Oxford), see pages 127-152, especially pages 139-140.	1-64
Y	US, A, 4,551,433 (DE BOER) 05 NOVEMBER 1985, see entire document.	1-18
Y	W.S. REZNIKOFF et al., "MAXIMIZING GENE EXPRESSION" published 1986 by BUTTERWORTH PUBLISHERS (Stoneham, MA), see pages 1-33, especially pages 2-4.	1-64
Y	JOURNAL OF BACTERIOLOGY, Volume 170, No. 11, issued November 1988, G. Sawers et al., "Anaerobic Regulation of Pyruvate Formate-Lyase from Escherichia coli K-12", pages 5330-5336, see entire document.	11, 31, 51, 53-64

* Special categories of cited documents:[15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search[2]	Date of Mailing of this International Search Report[2]
17 MARCH 1992	30 MAR 1992
International Searching Authority[1]	Signature of Authorized Officer[20]
ISA/US	Nancy Treptow Vogel ebw

Form PCT/ISA/210 (second sheet)(May 1986) B

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

Y	EUROPEAN JOURNAL OF BIOCHEMISTRY, Volume 177, issued October 1988, W. Rodel et al., "Primary Structures of Escherichia coli Pyruvate Formate-lyase and Pyruvate-formate-lyase-activating Enzyme Deduced From the DNA Nucleotide Sequences", pages 153-158, see entire document.	11,31,51, 53-64
Y	APPLIED AND ENVIRONMENT MICROBIOLOGY, Volume 53, No. 10, issued October 1987, L.O. Ingram et al., "Genetic Engineering of Ethanol Production in Escherichia coli", pages 2420-2425, see entire document.	12-14,32-34, 52-54, 59-61

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE[1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers _, because they relate to subject matter (1) not required to be searched by this Authority, namely:

2. ☐ Claim numbers _, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out (1), specifically:

3. ☐ Claim numbers _, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING[2]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Search Authority did not invite payment of any additional fee.

Remark on protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category*	Citation of Document,[16] with indication, where appropriate, of the relevant passages[17]	Relevant to Claim No.[18]
Y	JOURNAL OF BACTERIOLOGY, Volume 169, No. 3, issued March 1987, T. Conway et al., "Promoter and Nucleotide Sequences of the Zymomonas mobilis Pyruvate Decarboxylase", pages 949-954, see the entire document.	12-16,32-36, 52-54, 59-64
Y	ARCHIVES IN MICROBIOLOGY, Volume 144, issued June 1986, B. Brau et al., "Cloning and Expression of the Structural Gene for in Escherichia coli", pages 296-301, see the entire document.	12-16,32-36 52-54,59-64
Y	JOURNAL OF BACTERIOLOGY, Volume 171, No. 11, issued November 1989, K. Alam et al., "Anaerobic Fermentation Balance of Escherichia coli as Observed by In Vivo Nuclear Magnetic Resonance Spectroscopy", pages 6213-6217, see entire document.	15,16, 35, 36, 55, 56, 62, 63
Y	JOURNAL OF BACTERIOLOGY, Volume 171, No. 9, issued September 1989, C.M. Hamilton et al., "New Method for Generating Deletions and Gene Replacements in Escherichia coli", pages 4617-4622, see entire document.	19-64